

2017

Adult neurogenesis in a diurnal vertebrate: from hours to years

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BOSTON UNIVERSITY
SCHOOL OF MEDICINE

Dissertation

**ADULT NEUROGENESIS IN A DIURNAL VERTEBRATE:
FROM HOURS TO YEARS**

By

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Submitted in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

2017

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DEDICATION

I would like to dedicate this work to my parents, Maria and John, and my sister Emily.

ACKNOWLEDGMENTS

I am so grateful for the help and support of so many people that has led to the completion of this dissertation. First, I would like to thank all the member of the Zhdanova lab, past and present, who have been an essential part of my graduate career. To my mentor Dr. Irina Zhdanova, I am so appreciative of you for providing me with the initial opportunity to work in the lab as an undergraduate and continuing to believe in me throughout my career as a PhD candidate. Your enthusiasm for research is infectious and I'm fortunate to have learned from someone so knowledgeable and dedicated. To my lab colleague and friend, Erin McGowan, for being a hardworking and committed scientist who I'm confident will succeed in the field.

To my committee members, Tarik Haydar, Doug Rosene, Hui Feng and James Monaghan, for their astute questions and commitment to making my research better.

I also wanted to thank my PhD friends who have undergone this journey with me and in parallel. Their help with my research was invaluable and I'm proud to call them as some of my closest friends, Nadine Aziz, Joe Goodliffe, and Obi Onochie.

I want to thank my parents, Maria and John, and sister, Emily for their continued support and love. I also want to thank my girlfriend, Suzy, for her encouragement and Adobe expertise.

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ABSTRACT

The loss of neurons throughout aging, due to trauma or neurodegenerative diseases, was considered irreversible until recent discoveries demonstrated the capacity for the postnatal mammalian brain to generate new neurons in discrete niches capable of integrating into existing neural circuitry. Adult neurogenesis is highly dynamic and modulated by numerous factors. However, the temporal patterns of adult neurogenesis, kinetics of cell proliferation, and migration remain poorly understood. Zebrafish, a model used in this investigation, is a diurnal vertebrate with a circadian clock and clock-controlled processes organized similar to humans. Importantly, zebrafish display active neurogenesis.

The studies comprising this dissertation demonstrate for the first time that a diurnal vertebrate displays robust circadian, i.e. near-24-h, patterns of adult neurogenesis. It proceeds as an orderly transition of cells from G1 to S phase of the cell cycle throughout the day, followed by nighttime progression of G2 phase, culminating with M phase in the early morning. While all five neurogenic niches studied reveal a common circadian pattern, each niche demonstrates a distinct S length and timing of the G1-S transition.

Further investigation into kinetics of adult neurogenesis focused on the events occurring in the neurogenic niches over several days. Both experimental and mathematical modeling approaches determined a consistent number of neural stem cells (NSCs) dividing daily. These approaches also elucidated the predominant modes of division for transient amplifying cells, the neural progenitors (NPCs), the pace of migration and survival of their progeny.

Finally, this dissertation addressed age-related changes in adult neurogenesis in zebrafish, supporting its gradual decline with age. Developing a pathological aging model, based on excessive nutrition throughout development and maturation, revealed a major decline in the number of dividing NSCs and extreme modification of the pattern of division and survival of NPCs.

Together the results of the studies presented in this dissertation reveal that adult neurogenesis undergoes predictable dynamic changes over hours, days, and years. Future studies using a high-throughput zebrafish model should provide needed insights into the role of specific factors in adult neurogenesis and help develop therapeutic strategies to benefit human patients.

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LIST OF ABBREVIATIONS

AD.....	Alzheimer's Disease
ANP	Amplifying Neural Progenitor Cell
BrdU.....	Bromodeoxyuridine
CDAK	Cyclin Dependent Activated Kinase
CDC	Cell Division Cycle
CDK	Cyclin Dependent Kinase
CNS.....	Central Nervous System
Cry.....	Cryptochrome
D.....	Dorsal Telencephalon
DL.....	Lateral Pallium
DM	Medial Pallium
EdU	Ethynyldeoxyuridine
G0.....	Quiescence
G1	Gap 1
G2.....	Gap 2
GB.....	Glioblast
HD.....	Huntington's Disease
M-Phase	Mitosis
NB.....	Neuroblast
NPC.....	Neural Progenitor Cell
NSC.....	Neural Stem Cell

PD	Parkinson's Disease
Per	Period
PSA-NCAM	Polysialylated Neural Adhesion Molecule
RGC	Radial Glial Cell
RMS	Rostral Migratory Stream
S-Phase	DNA Replication
SC	Stem Cell
SCN	Suprachiasmatic Nucleus
SGZ	Subgranular Zone
SVZ	Subventricular Zone
Te	Telencephalon
VZ	Ventricular Zone
ZT	Zeitgeber Time

CHAPTER ONE

Introduction to the Topic of the Investigation

1.1 Adult Neurogenesis: an unexpected universal phenomenon of high significance

For years, the field of neuroscience firmly accepted Ramón y Cajal's theory that "no new neurons are added to the adult mammalian brain" postnatally and that it remains structurally constant (Ramón y Cajal, 1928). The landmark studies documenting the existence of neuronal proliferation in rodents (Altman and Das, 1965) and confirmation of neuronal fate in the dentate gyrus and olfactory bulb by electron microscopy (Kapan and Hinds, 1977) laid the foundation for further investigation.

Decades later, it is well established that adult neurogenesis is a universal phenomenon among vertebrates and in some invertebrates, involving cell proliferation, maturation, migration and integration into existing neuronal circuitry. Though the magnitude of this phenomenon is highly variable across species, there is evidence of its existence in crickets (Cayre et al., 1994), crustaceans (Harzsch and Dawirs, 1996) and zebrafish (Zupanc et al., 2005), as well as higher vertebrates, including birds (Paton and Nottebohm, 1984), rodents (Gould et al., 1992), monkeys (Gould et al., 1999) and humans (Eriksson et al., 1998).

Investigation into the phenomenon of adult neurogenesis is important not only for basic biological research, but has major translational value for human health. The search on intrinsic and extrinsic factors that can modulate cell division cycle progression within

specific brain regions may lead to novel prophylactic and treatment strategies for brain traumas and neurodegenerative disorders, or promote successful cognitive aging.

1.2 Approaches to studying adult neurogenesis

There are three principal approaches to studying adult neurogenesis. This can be done either directly in humans, using animal models as a surrogate for humans and through mathematical modeling. There are obvious practical and ethical constraints that limit experimentation in humans, though important studies have been conducted to establish adult neurogenesis in humans (Eriksson et al., 1998). Given that humans share similar though not identical biology and physiology with other species, if the process under study is present in an animal other than human, this can be used as an experimental model. Knowledge acquired from experimental observations and manipulations in animal models could then be translated to humans. The major complexity of biological systems necessitates the use of not only experimental but also computational and mathematical modeling. The mathematical approach allows for accurate description of real-time events, their kinetics and dynamics, and prediction of the effects of specific perturbations applied to the system. In combination with refined experimental techniques, mathematical modeling became a powerful tool of modern biology.

Determining the appropriate animal model for investigation into adult neurogenesis is essential. The majority of research is conducted in mammalian models, primarily in mice, rats and to a much lesser extent in monkeys (Fig. 1). However, for biomedical research, teleosts provide a number of exceptional advantages. Teleosts, the most popular

being zebrafish, were classically used as a developmental model due to their large progeny, short maturation time and external fertilization, with transparent embryos and larvae. Mapping of the zebrafish genome (orthologs for 70% of human genes) and the ease of genetic manipulations led to the high-throughput development of mutant and transgenic lines (Howe et al., 2013).

Importantly for this dissertation, zebrafish have remarkably active neurogenesis. Each day, thousands of neural stem/progenitor cells in adult zebrafish are undergoing cell division in 16 neurogenic niches spread throughout all subdivisions of the central nervous system, with the majority of newborn cells eventually differentiating into specialized neurons (Chapouton et al., 2007; Grandel et al., 2006; Zupanc et al., 2005; Kaslin et al., 2009).

1.3. The Process of Adult Neurogenesis: from neural stem cell to neuron.

There are major similarities in adult neurogenesis between species, though some notable differences exist as well. While describing here the main principles and components of adult neurogenesis, we will also provide a comparison between two species, the currently most popular mammalian model, mouse, and the subject of the investigation described in this dissertation, a teleost model, zebrafish.

During development, the neural tube of all vertebrates consists of a single layer of pseudostratified neuroepithelial cells with apicobasal polarity along their axis. In the

developing brain, these neuroepithelial cells initially undergo symmetric division amplifying the pool of embryonic stem cells (SCs). As the epithelium thickens, these neuroepithelial cells elongate and convert to radial glial cells (RGCs) with apicobasal polarity. RGCs begin a series of asymmetric divisions maintaining the stem cell pool while generating neurons directly or by generating neural progenitor cells (NPCs) which further amplify to generate neurons. This is followed by asymmetric cell divisions to generate glia: astrocytes, oligodendrocytes and ependymal cells. The products of these divisions comprise the central nervous system (CNS). In mammals, RGCs are retained postnatally to act as neural stem cells (NSCs), residing and dividing in specific regions of the brain, called the neurogenic niches. In zebrafish, a similar transition occurs in some neurogenic niches (e.g., telencephalic), while in other adult NSCs can retain neuroepithelial markers (e.g., cerebellar niche).

Neurogenesis persisting in the postnatal brain depends on stem/progenitor cell populations that have been specified during embryonic development. The distribution, location and extent of adult neurogenesis varies across vertebrate species. The two attributes that distinguish adult NSCs from their embryonic counterparts are the acquisition of quiescence (non-cycling) and their location in discrete cellular niches (Urban and Guillemot, 2014). Embryonic RGCs have a high proliferative rate, while adult RGCs remain in quiescence in G0 phase for long periods of time. This quality is essential for NSCs to maintain homeostasis and to avoid neural stem cell exhaustion.

NSCs are also believed to remain quiescent in order to withstand metabolic stress and preserve SC integrity by limiting mutations (Bond et al., 2015).

Stem cells are defined by their ability to self-renew and generate different cell types. When isolated and raised *in vitro*, cells collected from different neurogenic niches of adult rodent brains have shown the ability to expand and differentiate into cells of all three lineages (neurons, astrocytes and oligodendrocytes) following administration of growth factors (Palmer et al., 1997; Reynolds and Weiss, 1992). Furthermore, when lineage-restricted neural progenitors are exposed to different growth factors *in vitro* they acquire stem-like properties and are capable of differentiating into neurons and glia, though this is not seen *in vivo* (Gabay et al., 2003; Kondo and Raff, 2000; Palmer et al., 1999). In grafting studies, when cells isolated from the rat hippocampus were grafted into the rostral migratory stream they gave rise to neurons with specific neurotransmitter phenotypes, expressing tyrosine hydroxylase (dopaminergic cell marker). Those are not the cells normally generated in the hippocampus, but rather produced in the olfactory bulb, i.e., originate from SVZ (Suhonen et al., 1996). Moreover, in a technique known as heterochronic parabiosis (old mice surgically connected to young mice via their circulatory systems) can affect the systemic milieu which can inhibit or promote adult neurogenesis in an age-dependent fashion (Villeda et al., 2011). Together, this suggests the niche specific milieu, including cell-cell communication, extracellular matrices and humoral factors, rather than some NSC-specific lineage qualities promoting or limiting differentiation into particular cell types.

1.4. Adult Neurogenesis in Mammals

In mice, as in other mammals studied so far, there are two principal neurogenic niches, the subventricular zone (SVZ) and the subgranular zone (SGZ). Each of these regions contains niche-specific NSCs. Several reports also indicate a potential for adult neurogenesis in the mammalian hypothalamus (Evans et al., 2002; Xu et al., 2005; Cheng, 2013). The adult SVZ is located under an ependymal cell layer contacting the ventricle. GFAP- and Nestin-positive radial glia-like NSC's (type B cells), surrounded by a pinwheel of ependymal cells, extend an apical process with a primary cilium going through the ependymal layer to contact cerebrospinal fluid (CSF) and a basal process that terminates on an adjacent blood vessel. Type B cells generate transient amplifying progenitors (type C cells). Type C cells divide a few times before becoming neuroblasts (type A cells), which form a chain and migrate through the rostral migratory stream (RMS) to differentiate into different interneurons subtypes. More specifically, the neuroblasts differentiate into inhibitory GABA-ergic interneurons in the granule cell layer of the olfactory bulb as well as GABA-ergic, glutamatergic and dopaminergic interneurons in the periglomerular layer (Lledo et al., 2008)

Similar to the SVZ niche, GFAP-positive radial glia-like (RGC's or Type 1 cells) NSCs are present in the SGZ. They are located between the granule cell layer and hilus giving rise to intermediate progenitor cells (IPC's or Type 2 cells). IPCs undergo limited proliferation before generating neuroblasts which develop into immature neurons and migrate into the granule cell layer to differentiate into excitatory-glutamatergic dentate

granule neurons. Accordingly, adult mouse brain generates two different sets of differentiated cells: SVZ NSCs give rise to olfactory bulb interneurons and corpus callosum oligodendrocytes, while SGZ NSCs replenish dentate granule neurons and astrocytes (Bond et al., 2015).

1.5. Adult Neurogenesis in Zebrafish, in comparison to mammals

Similar to mammal, the brain of adult zebrafish contains discrete proliferative zones that house heterogeneous NSCs populations differentiating into distinct cellular fates. Unlike in mammals, however, the zebrafish brain has 16 distinct neurogenic niches, located along the ventricular walls or their remnants (Fig. 2). In the studies outlined in this dissertation, we have documented adult neurogenesis in 5 of these niches. Three of those are described here in more detail, for reasons of homology to mammalian neurogenic regions (dorsal and ventral pallium) or abundant proliferation (cerebellum).

The telencephalon (Te) of teleost, like that of mammals, is comprised a dorsal Te, or pallium, and ventral Te, or subpallium. The lateral domain of the zebrafish pallium (Dl) has been suggested to be homologous to mammalian hippocampus, while its medial domain (Dm) to contain amygdala-like nuclei (Than-Trong and Bally-Cuif, 2015). Due to telencephalic evagination in teleost (in contrast to invagination in mammals), part of their NSCs end up on the surface of the dorsal Te (D). This and visual transparency of zebrafish at larval stage allows for a unique *in vivo* imaging of these cells under confocal microscope (Barbosa et al., 2015). NSCs in the pallium are distinguished by either the

radial glial marker, GFAP (while also positive for Vimentin and S100B) and long radial processes reminiscent of mammalian embryonic radial glial cells (Adolf et al., 2006; Kizil et al., 2012). The actively cycling GFAP-negative cells in this region are likely to represent the intermediate neural progenitor cells (NPCs) or neuroblasts (Kaslin et al., 2008; Marz et al., 2010). Similar to that in mammals, a few days following division, daughter cells start expressing early neuronal differentiation marker HuC/D, increasing such expression over the next 2-3 weeks, with the cells reaching maturity after 4-8 weeks post-division (Adolf et al., 2006; Rothenaigner et al., 2011). The precise lineage of NSCs leading to neuronal subtypes has yet to be fully elucidated, but some have been recognized as parvalbumin- and calbindin- positive interneurons, in addition to GABAergic and glutamatergic neurons (Than-Trong and Bally-Cuif, 2015).

In contrast to pallium, the NSCs in the subpallial region of zebrafish brain express neuroepithelial characteristics (nestin expression) and do not express GFAP. There is a distinct similarity between the subpallial region of zebrafish brain and SVZ in mammals (Adolf et al., 2006). Part of the homology is reflected in cells of both structures being positive for the polysialylated neural adhesion molecule (PSA-NCAM), a marker for immature neuronal-committed progenitors, similar to neuroblasts in the SVZ, and the ability of cells to migrate to the olfactory bulb (Grandel et al., 2006). The majority of cells differentiate into neurons, with some of them, similar to that in the mammalian SVZ, become GABAergic and dopaminergic interneurons (Adolf et al., 2006, Mueller and Guo, 2009).

In the cerebellum, NSCs are found along the IVth ventricle (ventral) known as the ventricular zone (VZ) and ventricular recess (dorsal) displaced deep in the tissue, known as the upper rhombic lip (URL). These cells are polarized neuroepithelial cells positive for stem cell markers nestin and sox2, but are negative for radial glial markers GFAP, Vimentin, BLBP (Kaslin et al., 2009). A subpopulation of cells with radial glial morphology and immunopositive for GFAP, vimentin and BLBP, but not S100B did not colocalize with proliferation markers (Kaslin et al., 2009). These cells are thought to act as scaffolds for the migrating neuroepithelial cells. The neuroepithelial stem cells give rise to dividing intermediate progenitors which migrate dorsolaterally and differentiate into excitatory granule neurons utilizing glutamate and a limited number of glia (Bergmann glia-like cells) and inhibitory interneurons utilizing GABA or glycine (Kaslin et al, 2013).

1.6. Different Modes of Division for Neural Stem and Neural Progenitor Cells

It is widely accepted that NSCs are maintained for long periods of time, some throughout life, and divide infrequently, having prolonged periods of quiescence. A number of studies suggest NSCs are capable of several modes of division, including symmetric and asymmetric or direct conversion (Bonaguidi et al., 2011) (Fig. 3 a-e). Expansion of NSCs is accomplished by a symmetric division into two identical NSCs, increasing the overall NSC pool (Fig. 3a). Their maintenance can be accomplished through two modes of asymmetric division, each producing one NSC and either one transient amplifier, a neural progenitor cell (NPC), or one post-mitotic neuroblast/glioblast (NB/GB) (Fig. 3 b,c).

Unlike that, the other modes of division/conversion cause a depletion of NSC pool, due to NSC either dividing into two NPCs or two NBs/GBs (Fig. 3 d,e) or direct conversion into one NPC or NB/GB (Fig. 3 f,g). Under normal circumstances, the asymmetric NSC division (Fig. 3b), maintaining NSC and creating NPC, appears to be most probable (Barbosa et al., 2015). A conversion of NSC to NB/GB appears to be the least probable event, suggesting urgent need for brain repair following trauma or another pathological condition (Barbosa et al., 2015; Bonaguidi et al., 2011).

Unlike NSCs, NPCs are short-lived transient amplifying cells, capable of several divisions. Those divisions can also be symmetric, producing two NPCs or NBs/GBs (Fig. 4 a,b). An asymmetric division leads to the maintenance of NPC and production of a neuroblast (Fig. 4c). The final mode of division is a direct conversion to NB/GB, depleting the NPC cell pool. The main critical period for newborn cell survival occurs early in the first 1-4 days after cells birth where up to 60% of cells undergo apoptosis during the transition from NP to NB/GB and a second smaller critical period during the first 1-3 weeks after birth (Sierra et al, 2010). Thus, the majority of apoptosis occurs when cells are highly proliferative and those that make it past this phase successfully integrate anatomically and functionally into the existing circuitry.

1.7. Cell cycle regulation

Investigation into the phases of the cell division cycle (CDC) in NSCs and NPCs involved in adult neurogenesis offers another way of evaluating this complex process and defining its vulnerabilities by the effects of different factors or disorders. Across all

eukaryotes, CDC is divided into four sequential phases: Gap 1 (G1), S-phase (DNA synthesis), Gap 2 (G2) and M-phase (mitosis, cell division). After mitosis, the cell can either re-enter a new cell cycle by initiating G1 or interrupt proliferative activity and reside in a quiescent state known as Gap 0 (G0). The cell enters G1 through perceiving certain extracellular factors (e.g., growth factors) or detecting the availability of nutrients. G1 is characterized by cell growth and preparation of machinery for DNA synthesis. Transition from G1 to S-phase involves an important check-point, known as the restriction point, during which the cell will exit the cell cycle if the ideal conditions are not met. If the cell successfully passes this check point, it initiates DNA synthesis (S-phase), resulting in DNA replication. This is followed by continued cell growth in G2 phase and preparation for M-phase. Following another G2/M check-point, the CDC culminates with cell division.

These complex phases of CDC are precisely orchestrated by the changes in the activity of cyclin-dependent kinases (CDKs). On their own, CDKs remain inactive, but when they partner with regulatory protein subunits, called cyclins, named for their periodic synthesis and degradation during specific phases of the cell cycle, they become Cyclin-Dependent-Activated kinases (CAKs). CAKs allow phase-progression and transition of the cell cycle through phosphorylation of target proteins. Cyclins are then degraded, causing inactivation of their complementary CDK. In mammals, Cyclin D-CDK4/6 complex is responsible G1 progression, Cyclin E/CDK2 allows for G1-S transition, Cyclin A/CDK2

is necessary for S-phase progression, and Cyclin A/B/CDK1 for entry into and maintenance of M-phase (Sherr and Roberts, 2004).

The cell cycle is negatively regulated during any phase of the cell cycle by two families of non- enzymatic cyclin-dependent kinase inhibitors (CDKIs) that interact with already formed cyclin/CDK complexes: Ink4/ARF type (p16, p15, p18, and p19) and Cip/Kip type (p21, p27, and p57) (Pechnick et al., 2008). The specific role of all of these regulators in adult neurogenesis remains to be fully understood. A recent study suggested that p27Kip1, a regulator of G1, can keep NSCs quiescent and out of the cell cycle (Andreu et al., 2015). Further understanding of how NSC proliferation and differentiation are regulated by the cell cycle constituents during adult neurogenesis should offer new therapeutic approaches to neurodegenerative disorders and brain trauma.

1.8. Adult neurogenesis regulation by intrinsic and extrinsic factors

Adult neurogenesis is a dynamic process and each of its three phases, including cell proliferation, differentiation and survival, can be modulated and regulated by a variety of intrinsic and extrinsic factors (Fig. 5). Intrinsically, growth factors, neurotransmitters, neurotrophins, cytokines, transcription factors and hormones regulate adult neurogenesis (for review, Urban and Guillemot, 2014). For instance, in the SVZ, GABA receptor activation suppresses NSC cell proliferation (Fernando et al., 2011), while acetylcholine can promote the proliferation of NSCs in the SGZ (Itou et al., 2011). Similarly, on the organismal level, factors including voluntary exercise or learning have been shown to increase neurogenesis (Gould et al., 1999; van Praag et al., 1999).

Extrinsically, neurogenesis can be also modulated by multiple factors. Those typically affect the entire organism and thus it could be more difficult to differentiate cause-effect relationship of these factors to the state of adult neurogenesis. Adverse extrinsic factors can downregulate neurogenesis directly or through inducing stress responses (for review, Aimone et al., 2014). In contrast, voluntary exercise can increase SGZ cell proliferation, while exposure to an enriched sensory and social environmental stimulation can promote the survival of new neurons (Kee et al., 2007, Tashiro et al., 2007).

A healthy lifestyle, including proper diet, can slow the aging process and decrease the risks of age-related diseases. While starvation is detrimental, moderate reduction in caloric intake or increased intervals between meals can have positive effects on physiological functions, and were found to slow down aging, increase lifespan, and counteract age-related diseases (for review, Sohal and Weindruch, 1996; Anson et al., 2003; Bishop and Guarente, 2007). Moreover, dietary restriction (20-40% reduction in food consumption) in rodents can increase adult neurogenesis and this is attributed to the improved survival of newly generated cells (Lee et al., 2000; Lee et al., 2002, Lee et al., 2002a). Studies suggest that diverse metabolic factors can modulate adult neurogenesis, including high fructose or fat diets, as well as metabolic disorders such as diabetes, metabolic syndrome, obesity (Bruce-Keller et al., 2009; Uranga et al., 2010; Van der Borght et al., 2011, Kanoski & Davidson, 2011; Farooqui et al., 2012; Nose-Ishibashi et al., 2014).

Pharmacological agents present in the environment, including pollutants or drugs, can adversely affect adult neurogenesis. For example, drugs activating GABA were shown to reduce adult neurogenesis (Wu and Castren, 2009). In contrast, antidepressant medications have been recently found to promote adult neurogenesis in animal models (in review, Balu and Lucki, 2009). Indeed, several classes of antidepressants have been shown to increase hippocampal neurogenesis in rodents (Malberg et al., 2000; Manev et al., 2001). Furthermore, the delay in the onset of the therapeutic effects of antidepressants is correlated with the maturation time of newly generated hippocampal neurons (Sahay and Hen, 2007). All this evidence suggests that neurogenesis might in fact be part of etiology and pathogenesis of depression. Decrease in the number of activated NSC in the hippocampus of patients with schizophrenia also suggested that NSC deficiency might contribute to the pathogenesis of this disease (Reif et al., 2006).

Aging is associated with decline in neurogenesis, as described in detail below (Section 1.12). Pathologic aging further augments the loss of neurogenesis. Currently, multiple studies are addressing whether deficits in adult neurogenesis play a role in the pathophysiology of diverse neurodegenerative disease states. Alterations in adult neurogenesis are documented in all neurodegenerative diseases, such as Parkinson's Disease (PD), Alzheimers Disease (AD) and Huntington's Disease (HD), along with similar impairments in the animal models of these disorders (Winner and Winkler, 2015). For instance, in a mouse model of AD, animals had a decreased ability to

generate new neurons in the SGZ, and this positively correlated with the presence of A β plaques in the hippocampus (Rodriguez et al., 2008).

Not only may research on neurogenesis lead to novel prophylactics of different brain conditions, but increasing the rate of NSC proliferation and survival of their progeny could reduce the development and progression of major human diseases.

1.9. Circadian regulation of the cell cycle

The majority of physiological and behavioral processes are cyclic, with periods ranging from milliseconds to annual. One of the most robust biological rhythms has a period close to 24-h, and is called “circadian” (from Lat. for “near day long”). The rhythm has evolved in direct response to the planetary movement and thus a 24-h period of environmental illumination. In the organisms, including humans, the circadian rhythms are endogenous, being present in virtually every cell of the body. A master clock located in the suprachiasmatic nucleus (SCN) of the hypothalamus provides a unifying signal for the entire body. The period of SCN oscillation however differs between individuals, ranging from around 23 to 25h. Notably, the SCN rhythm can entrain to the a range of circadian periods, providing adaptive entrainment to the 24-h day.

The molecular organization of the circadian clock is well-conserved, and it is based on translational-transcriptional mechanisms that drive the circadian rhythm at the cellular level (Reppert and Weaver, 2002). The clock is cell autonomous and consists of a series

of transcription-translation feedback loops in which the positive arm (Clock and Bmal proteins) drives the expression of the negative arm (Period (Per) and Cryptochrome (Cry)) that, in turn, feedback to down-regulate their own expression and to allow for the start of a new cycle (Welsh et al., 2010). In addition to these, multiple other transcription factors modulate the circadian clock in a tissue and cell specific manner (reviewed in, Reppert and Weaver, 2002). Even though the clockwork has been well studied in a number of models, the mechanisms by which the circadian machinery influences the cell processes is only partially understood.

The downstream processes these clocks control including, biochemical, physiological and behavioral occur within a similar circadian period. The most notable example is the sleep-wake cycle, though cardiovascular, immune, metabolic or cognitive processes are also under a tight control of the circadian clock. This dissertation is devoted to the investigation of the circadian regulation of the adult neurogenesis. As a first step, it thus addresses the role of the internal clock in the cell cycle progression.

The CDC is a highly regulated process, since any error can produce a dangerous progeny, leading to tumorigenesis. The precise timing of events might thus be essential for the successful division, creating two viable daughter cells. Moreover, the circadian time at which the cell divides might define its further fate. This is because the majority of factors affecting neurogenesis are under the clock control. Moreover, precise timing of the cell cycle provides temporal coordination of cell proliferation in diverse tissues and organs, as

was demonstrated in a range of species including humans (Smaaland, 1996; Yang, 2010; Bjarnason et al., 2001; Janich et al., 2014).

The clock may regulate cell proliferation machinery directly, potentially through products of the core clock genes serving as transcription factors and thereby affecting the cell cycle. Alternatively, the clock can regulate CDC through periodic modulation of the immediate cell environment. The *in vitro* single-cell or tissue models provide unique insights into the basic mechanisms of interactions between the cell division cycle and circadian clock (Bjarnason and Jordan, 2002; Matsuo et al., 2003; Dekens et al., 2003; Feillet et al., 2015; Malik et al., 2015). Furthermore, models with genetic manipulations of the molecular circadian clock display abnormal or disrupted cell proliferation (Bouchard-Cannon et al., 2013; Rakai et al., 2014). Together, this further supports a major role of the circadian clock in CDC.

1.10. The Diverse Time Scales of Adult Neurogenesis: HOURS

As part of our investigation into the role of timing in adult neurogenesis, we addressed different time scales, from hours of the circadian period to years of life. The question of whether adult neurogenesis might be under control of the circadian clock has been addressed in several studies. However, these studies have led to inconsistent results. Some studies in lobsters (Goergen et al., 2002), mice (Bouchard-Cannon et al., 2013) and rats (Guzman-Marin et al., 2007) found the number of cells in S-phase increases at day-night transition or, in mice, mitosis peaks at night (Tamai et al., 2008). Others found no such daily variation in S-phase in mice (Kochman et al., 2006; Van Der Borght et al.,

2006; Tamai et al., 2008, Ponti et al., 2013) and rats (Ambrogini et al., 2002) or variation in mitosis in mice (Ponti et al., 2013).

Remarkably, all the above mentioned studies have been conducted in nocturnal species and the work presented in this dissertation and corresponding publications is the first to date to report circadian clock regulation of adult neurogenesis in a diurnal species. To appreciate how translational research can significantly benefit from studying the circadian control of adult neurogenesis in animals that, similar to humans, are diurnally-active requires understanding the way the clock controls physiological and behavioral functions in a species-adaptive manner.

The highly conserved core circadian clock mechanisms, including clock gene expression, the daytime activity of SCN neurons or nighttime production of the principal circadian hormone, melatonin, all follow similar temporal patterns in nocturnal and diurnal animals. However, the downstream processes these clocks control, for example, a diurnally active human and a nocturnally active mouse, are inverted, having a 12-hour phase difference. This is reflected in such behavioral states as wakefulness (vs. sleep), peaks cognitive performance, cardiovascular and metabolic functions occurring at daytime in humans and at night in mice. For these reasons, for translational circadian research, it is optimal to use a diurnal vertebrate model with circadian patterns similar to those in humans (Zhdanova et al., 2001). This includes the need for understanding temporal patterns of adult neurogenesis in a diurnal vertebrate, comprising one of the principal aims of this dissertation.

1.11. The Diverse Time Scales of Adult Neurogenesis: DAYS

After establishing a circadian pattern of adult neurogenesis in a diurnal vertebrate, we characterized temporal patterns of maintenance and turnover of cells critical for adult neurogenesis, the NSCs and NPCs, involving several days. Similarly, we explored the daily rate of migration of postmitotic neuroblasts/glioblasts from a neurogenic niche in an adult vertebrate brain.

Uncovering the fate and frequency of the division patterns, in addition to the debate about the self-renewal capacity and multipotentiality of adult neural stem cells, is a subject of ongoing research (Ming and Song 2011; Bond et al., 2015). Despite the known stem cell model describing repeated self-renewal followed by quiescence, one seminal study put forth an alternate “disposable” stem cell model to describe events in the young mouse dentate gyrus (Encinas et al., 2011). In this model, a quiescent neural progenitor (QNP) becomes activated and undergoes approximately 3 rounds of asymmetric division generating one amplifying neural progenitor cell (ANP) per division. It is further suggested that each ANP undergoes 2 rounds of symmetric division generating 4 ANPs which are converted into 4 neuroblasts (NBs). The NBs differentiate, totaling 16 NB per QNP. After these 3 rapid divisions, the QNP begins acquiring astrocytic morphology resulting in a depletion of the stem cell pool. Similarly, in the zebrafish dorsal telencephalon, homologous to the mammalian SGZ, Barbosa et al. (2015) documented that NSCs can also be depleted through lifetime. In fish, both symmetric and asymmetric divisions expanding or maintaining the NSC pool have been observed. Also, a portion of

NSCs can convert directly into neuroblast and then neurons, depleting the NSC pool with age. In further support of Encinas et al. (2011), Barbosa showed progenitor cells divide once or twice before terminally differentiating into neurons (Barbosa et al., 2015). In contrast, Bonaguidi et al., (2011) showed active NSC self-renewal capacity in the mouse dentate gyrus. This study described four fates for NSCs: symmetric self-renewal division, asymmetric division to generate a PC, asymmetric division to generate an astrocyte and NSC, or direct differentiation to astrocyte. Furthermore, they describe robust division potential for NPCs, up to 5 cycles.

In the mouse SVZ, data collected by Ponti et al., (2011) also support the notion of a self-renewing NSC based on the investigation of the frequency of division and cell fates. Activated B1 cells give rise to transit-amplifying precursors (C cells) that divide symmetrically approximately three times before becoming migratory neuroblasts (A cells), which divide one or two more times while en route to the OB . While Calzolari et al., (2015) corroborates a rapidly expanding progeny, they document overall depletion of NSCs. Given the different labeling approaches and lack of specific NSC labels, these studies could be identifying different populations, and this might explain some discrepancies.

1.12. The Diverse Time Scales of Adult Neurogenesis: YEARS

Senescence is a process shared by all vertebrate species and is associated with gradual changes in physiological and cognitive functions. Aging can be characterized as “successful” in those individuals who retain their cognitive and physical capacities

throughout life span or “unsuccessful” in those who exhibit significant decline, often associated with age-related diseases, including Alzheimer’s and Parkinson’s Disease. A third class of individuals are those that age faster than their chronological age, known as premature agers. Factors including genetics, diet, stress, and lifestyle can have strong impact on the aging process. Adult neurogenesis, a lifelong proliferation of new neurons, may potentially be a sensitive variable to distinguishes aging phenotypes and an important therapeutic target to promote successful aging and prevent unsuccessful aging.

Not only do all vertebrate species exhibit neurogenesis, but zebrafish, rodents, non-human primates, and humans alike exhibit progressive age-related decline in neurogenesis (Kuhn et al., 1996; Gould et al. 1999; Leuner et al., 2007; Maslov et al., 2004; Heine et al., 2004; Aizawa et al., 2011; Knoth et al., 2010; Edelman et al., 2013).

There are several possible mechanisms responsible for the age-related decline in neurogenesis including: (1) increase in NSC quiescence, (2) loss of neural stem cells through death or differentiation, (3) decrease in NSC or NPC proliferation, (4) decrease in the survival of their progeny, or (5) reduction in neuronal fate commitment. Currently, it is considered that aging of a tissue is to large extent caused by a decline in the regenerative capacity of its resident stem cells. This could be a result of the progenitor cells failing to respond to stimuli to proliferate or due to age-related changes in the milieu of the neurogenic niche. Neurogenesis in aged animals can be restored to a certain extent by voluntary exercise, suggesting that these cells still have the capacity for responding to powerful stimuli (van Praag et al., 2005; Zhao et al. 2008). According to the “disposable”

stem cell model, NSCs might not only stop being mitotically active with age but then can terminally differentiate into post-mitotic astrocytes (Encinas et al., 2011). This is consistent with well-established increase in astrocytes with age. However, more research is needed to clarify the mechanisms involved in neurogenic aging.

Zebrafish is a promising model of aging, and our laboratory has contributed to the characterization of zebrafish aging. Zebrafish live for up to 6 years, on average about 3-4 years, and exhibit gradual senescence, similar to humans (Kishi et al., 2009). There is a physical phenotype of scoliosis with muscular atrophy with aging (Gerhard et al., 2002), development of cataracts (Kishi et al., 2008) and an increase in the senescence associated β -galactosidase (SA β -gal) aging biomarker (Kishi et al., 2008), to name a few.

Behaviorally, zebrafish show a decline in circadian rhythmicity and spatial memory with age (Yu et al., 2006). Aging in zebrafish is associated with a decrease in regenerative capacity of peripheral tissue (Tsai et al., 2007) and neurogenesis (Edelman et al., 2013; Barbosa et al., 2015). While Edelman et al. (2013) attributed the decrease in neurogenesis to a decline in NSCs cycling behavior, Barbosa et al. (2015) suggested the NSC pool was depleted. Further studies are required to better understand all the factors and mechanism in changes of adult neurogenesis with zebrafish aging.

Chapter 1.13 Tables and Figures

Figure 1. Phylogenetic tree showing the main animal models commonly used in biomedical research and their evolutionary relationships.

The divergence time, in millions of years (Mya), is based on multiple gene divergence and protein divergence studies (Wheeler and Brandi, 2009).

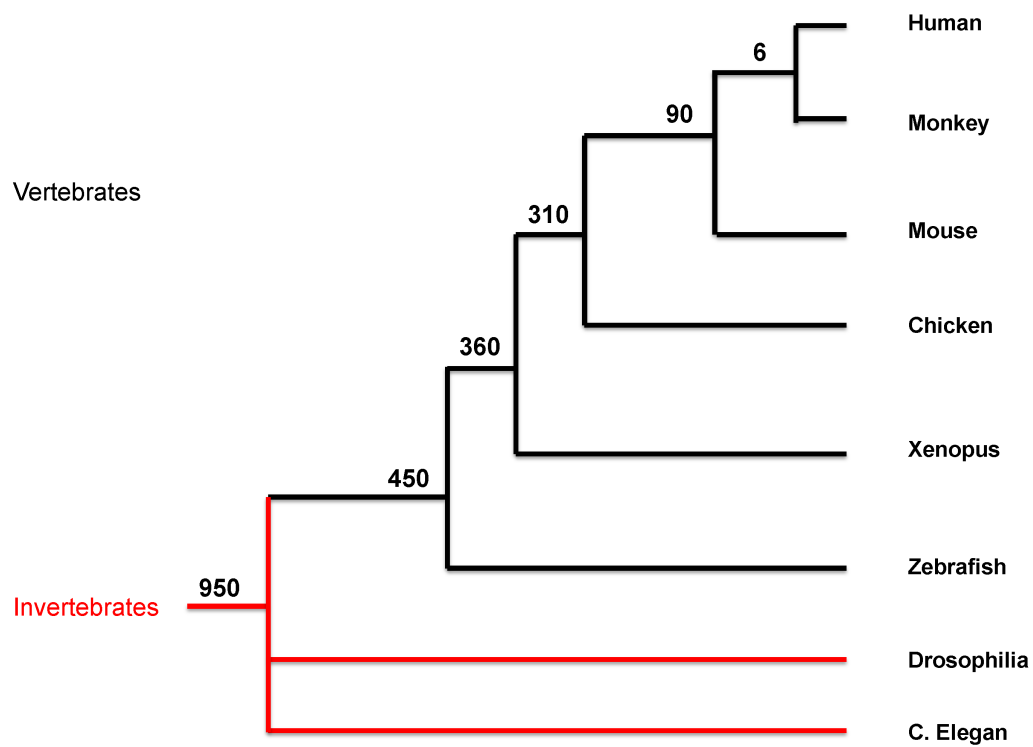


Figure 2. Adult neurogenic niches in the vertebrate models: mouse versus zebrafish (Kizil et al., 2012).

Mouse brain (left) has 2 well delineated neurogenic niches, subgranular zone of the hippocampal dentate gyrus and the subventricular zone of the lateral ventricles, versus 16 discrete niches in the zebrafish brain (right). Adult stem cell niches (red) and neurogenic niche (blue).

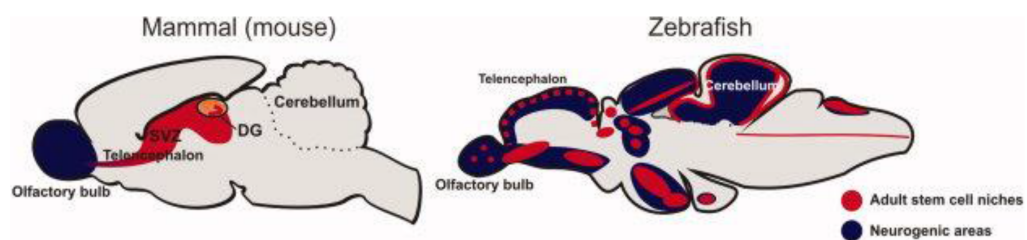


Figure 3. A schematic diagram depicting the distinct modes of NSC division in the neurogenic niche.

NSC's are capable of several modes of division: **A.** symmetric division expanding the NSC pool; **B,C.** NSC pool maintenance through symmetric division and creation of a NPC (**B**) or NB/GB (**B**); **D, E, F** depletion of the NSC pool through symmetric division into two NPCs (**D**), or two NB/GBs (**E**) or conversion into a NB/GB. Neural stem cell, NSC, (red circle); neural progenitor cell, NPC, (blue square); neuroblast/glioblast, NB/GB, green triangle.

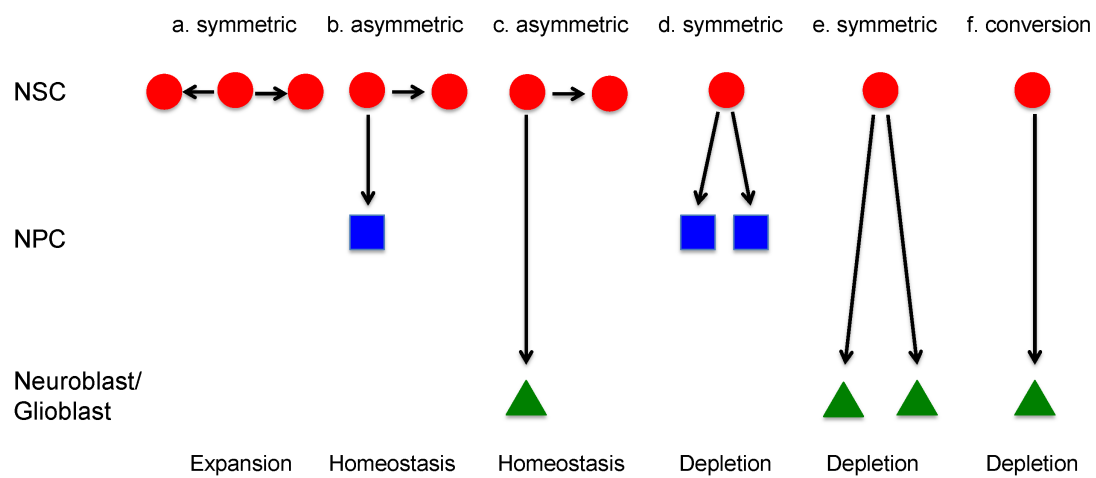


Figure 4. A schematic diagram depicting the distinct modes of NPC division in the neurogenic niche.

NPC's are capable of several modes of division: **A,B.** symmetric division expanding the NSC pool (**A**) or depleting the NPC and creation of two NB/GBs (**B**); **C.** asymmetric division resulting in homeostasis of the NPC pool and creation of a NB/GB; **D.** direct conversion of NPC to NB/GB depleting the NPC pool. Neural progenitor cell, NPC, (blue square); neuroblast/glioblast, NB/GB, green triangle.

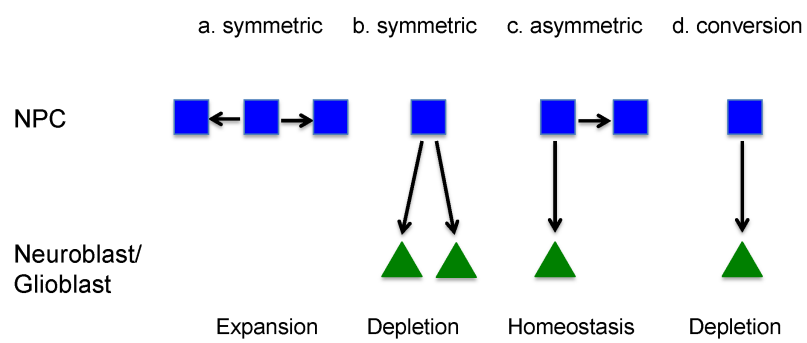
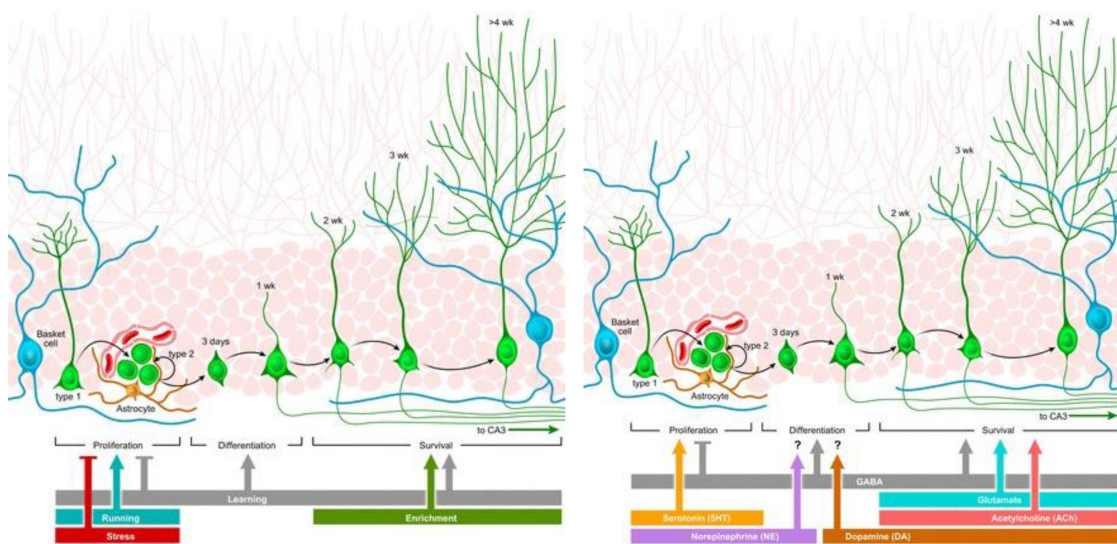


Figure 5. Intrinsic and extrinsic factors regulate mammalian adult neurogenesis at each of the three stages: proliferation, differentiation and survival (Aimone et al., 2014).

Regulation of neurogenesis by various behaviors (left) and network factors (right). These factors can effect all phases of adult neurogenesis by either enhancing (arrow) or suppressing (flat).



CHAPTER TWO: CIRCADIAN KINETICS OF ADULT NEUROGENESIS IN A DIURNAL VERTEBRATE

Published: Journal of Neuroscience 15 February 2017, 37 (7) 1900-1909;

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*V.A. and A.J.S. contributed equally to this work

Chapter 2.1: Abstract

The circadian system may regulate adult neurogenesis via intracellular molecular clock mechanisms or by modifying the environment of neurogenic niches, with daily variation in growth factors or nutrients depending on the animal's diurnal or nocturnal lifestyle. In a diurnal vertebrate, zebrafish, we studied circadian distribution of immunohistochemical markers of the cell division cycle (CDC) in five of the sixteen neurogenic niches of adult brain, the dorsal telencephalon, habenula, preoptic area, hypothalamus and cerebellum. We find that common to all niches is the morning initiation of G1/S transition and daytime S-phase progression, overnight increase in G2/M and cycle completion by late night. This is supported by the timing of gene expression for critical cell cycle regulators, cyclins D, A2, B2 and cyclin-dependent kinase inhibitor p20 in brain tissue. The early night peak in p20, limiting G1/S transition, and its phase angle with the expression of core clock genes, *Clock1* and *Per1*, are preserved in constant darkness, suggesting intrinsic circadian patterns of cell cycle progression. The statistical modeling of CDC kinetics reveals the significant circadian variation in cell proliferation rates across all of the examined niches, but inter-niche differences in the magnitude of circadian variation in CDC, S-phase length, phase angle of entrainment to light or clock, and its dispersion. We conclude that, in neurogenic niches of an adult diurnal vertebrate, the circadian modulation of cell cycle progression involves both systemic and niche-specific factors.

Chapter 2.2: Introduction

The seminal discovery that the adult brain produces new neurons capable of integrating into existing neuronal circuitry (Altman and Das, 1965; Kaplan and Hinds, 1977; Paton and Nottebohm, 1984) raised hopes that adult neurogenesis could help prevent and treat neurodegenerative and age-dependent brain conditions. However, efficacy of prospective treatments might significantly depend on their timing, if the integrating system of the endogenous circadian clock regulates the cell division cycle (CDC) in neuronal stem and progenitor cells. This is likely since tissue-specific clock-controlled genes are serving as key regulators of cell proliferation in diverse tissues (Fu and Kettner, 2013), and results of *in vitro* studies revealed clock coupling and modulation of cell proliferation in mammalian cells (Beiler et al, 2014; Janich et al., 2014; Feillet et al., 2015).

Whole animal models can further enhance understanding of the role of clock-controlled intrinsic endocrine and physiological factors that define daily changes in the immediate cell environment. The translational value of such models would largely depend on their temporal adaptation being similar to humans. This is because the core clock molecular mechanisms, including gene expression patterns, daytime SCN neuronal activation or nighttime surge in circulating melatonin are highly conserved. In contrast, the downstream clock-controlled functions in diurnal and nocturnal species have a 12-hour phase difference. This includes numerous behavioral, cognitive, genomic, enzymatic, metabolic and neuronal processes that occur in antiphase in, for example, nocturnal mice and diurnal humans. Moreover, the nature of CDC and its high-energy demands suggest

that prominent circadian organization of the sleep-wake and feeding cycles can be critical for the overall success of adult neurogenesis (Yamaguchi et al., 2013; Lee et al., 2014; Mueller et al., 2015). Thus, while considering a role for such periodic physiological functions in the production, survival and incorporation of new neurons into existing networks, translational goals favor diurnal species.

This report, to our knowledge, is the first one to address circadian control of CDC progression in neurogenic niches of a diurnal vertebrate. Earlier, the circadian patterns of adult neurogenesis were explored in nocturnal species. Some indicated an increased number of S-phase cells at day-night transition in the brains of lobsters, mice and rats (Goergen et al., 2002; Guzman-Marin et al., 2007; Bouchard-Cannon et al., 2013). Genetic manipulations of the molecular circadian clock were also found to disrupt cell proliferation in mice (Bouchard-Cannon et al., 2013; Rakai et al., 2014). Other studies, however, did not document daily variation in S-phase in nocturnal rodents (Ambrogini et al., 2002; Holmes et al., 2004; Kochman et al., 2006; van der Borght et al., 2006), even when mitosis peaked at night (Tamai et al., 2008).

Our choice of a diurnal vertebrate, the zebrafish, to study circadian control of adult neurogenesis in a whole animal is based on its robust circadian clock (Cahill, 1996; Whitmore et al., 1998), daytime feeding (Peyric et al., 2013) and prominent sleep-wake cycle (Zhdanova, 2001). Importantly, this species has remarkably active adult neurogenesis (Zupanc et al., 2005). Each day, thousands of cells in 16 neurogenic niches

of the adult zebrafish brain are undergoing division, with the majority of newborn cells eventually differentiating into specialized neurons (Zupanc et al., 2005; Grandel et al., 2006; Adolf et al., 2006; Kaslin et al., 2009, Kaslin et al, 2013).

Here we demonstrate circadian kinetics of CDC in neurogenic niches of an adult diurnal vertebrate, and its enhancement by the entrainment to environmental light-dark cycle. The pattern common to different neurogenic niches includes transition from G1 to S phase of CDC early in the day, with evening peak in the number of cells undergoing DNA replication, and nighttime transition through G2/M phases completed by early morning hours. The magnitude of circadian variation, phase angle of entry into S-phase and the mean S-phase length differ between the five neurogenic niches studied. Together, this suggests the role for both systemic and niche-specific factors in the temporal pattern of adult neurogenesis in a diurnal vertebrate.

Chapter 2.3: Materials and Methods

Animals

Adult male zebrafish (*Danio rerio*, wild-type AB strain), 12 $\pm$ 1 months old, were maintained on a 14 h light :10 h dark (14:10 LD) cycle, at 28°C, in 3L tanks of a multi-tank system (Aquaneering), as per standard practices (Whitmore et al., 2000). They were fed 3 times a day, at zeitgeber times (ZT) 1, 4 and 8 (ZT0= lights on time), with live brine shrimp supplemented with fish pellets (Lansy NRD). All animal procedures were

performed in accordance with Institutional Animal Care and Use Committee (IACUC) at Boston University School of Medicine.

5-bromo-2'-deoxyuridine (BrdU) Treatment

At 2 h or 4 h intervals over a 24 h period, individual groups (N=6 per 1 L tank) were treated with S-phase marker, BrdU (Sigma-Aldrich), with stock solution being administered directly into a 1L fish tank. The choice of this BrdU dose (6.5 mM) was based on a series of preliminary experiments, using a range of BrdU concentrations in tank water and times of exposure, and compared with intraperitoneal injection of 10 mM BrdU solution (1 μ l/100 mg body weight), as per earlier studies (Zupanc et al., 2005). Our preliminary data also indicated that a 1-h long immersion in 6.5 mM BrdU solution was sufficient to reliably label the brain cells in S-phase *in vivo*. It was taken into consideration that earlier zebrafish studies did not reveal any short-term toxicity of higher BrdU doses used for immersion (Byrd and Brunjes, 2001; Makantasi and Dermon, 2014; Laranjeiro et al., 2013), with this being supported by our survival studies in larval and adult fish (not reported here). We also relied on earlier reports that BrdU method is not sensitive enough to detect DNA repair, suggesting that BrdU-labeled cells represent a population undergoing S-phase of CDC (Palmer et al., 2000; Cooper-Kuhn and Kuhn, 2002). This was also supported by the presence of the BrdU⁺ cells only in the earlier identified neurogenic niches and not other regions of zebrafish brain where DNA repair might occur.

Palbociclib (PD 0332991) Treatment

A selective inhibitor of cyclin-dependent kinase (CDK) 4/6, PD 0332991 (Selleckchem.com), was administered directly into the tank water. The dose (2 μ M final concentration in tank water) was based on earlier reports of dose-dependence of the in vitro and in vivo effects of PD 0332991 suggesting efficacy for concentrations as low as 0.08 μ M and an exclusive G1 arrest being maintained at concentrations as high as 10 μ M (Fry et al., 2004), with cytotoxicity not observed at levels below 5 μ M (Barton et al., 2013). Fish were exposed to the drug for 8 h, from ZT 22 (Night 1) to ZT 6' (' = next Day), followed by a washout period until sample collection, to cover the entire period of G1/S transition in the neurogenic niches studied. To determine whether the drug acutely changes the expression of clock genes or genes involved in cell cycle progression, fish were collected during treatment (ZT3). To document the delayed consequences of PD 0332991 administration on CDC progression, fish were collected on Night 2, at ZT 19' or ZT 23' for the analysis of the expression of CDC regulators. For immunohistochemical (IHC) analysis of the effects of PD 0332991 on the number of cells undergoing S-phase, fish were treated with BrdU for 4 h prior to sample collection at ZT11' or ZT15' (Night 2), i.e., at the time of peak in S-phase pattern in all the neurogenic niches studied (N=5-6 per treatment-by-time group).

Evaluation of Intrinsic circadian rhythms in Constant Darkness

Fish were habituated within experimental groups (N=6 per 3 L tank) for one week in 14:10 LD and then transferred as a group into non-transparent 1 L tanks at the beginning

of the dark phase (ZT14 or beginning of Night 1). Fish were then maintained in constant darkness (DD), for 37 - 69 h, on a recirculating multi-tank system. Sample collection for the analysis of changes in mRNA abundance for clock genes and genes involved in cell cycle progression was initiated at ZT3' on subjective Day 2 (37 h in DD), and proceeded every 4 h for the next 32 h (i.e., until 69 h in DD). The matched control groups remained in LD and food was provided simultaneously to control and DD-exposed animals.

Immunohistochemistry

Fish were euthanized with an overdose of MS222 (200 mg/l) and heads were fixed overnight in 4% paraformaldehyde in phosphate buffered saline (PBS) at 4°C. Brains were then dissected out, cryoprotected in 30% sucrose/PBS, placed in embedding solution (O.C.T) and stored at -80°C, until cut. Coronal 20 µm sections were prepared using a cryostat (Microm HM505E) and placed onto Fisherbrand Superfrost Plus slides (Fisher), then stored at -80°C, until processed. The sections were washed in 0.1 M PBS and antigen retrieval was performed using both heat, incubated in 50% formamide / 50% 2X SSC at 65°C for 2 h, and acid, 2 M HCl at 37°C for 30 min and 0.1 M boric acid (pH 8.5) for 10 min at room temperature. Following a PBS wash, the slides were incubated overnight in primary rat anti-BrdU antibody (Accurate, 1:500; all antibodies in 0.1 M KPBS + 0.4% Triton-X100) at 4°C. Then, the slides were incubated for 1.25 h in secondary biotinylated rabbit anti-rat (Vector, 1:1000). After washing in PBS, chromogenic visualization was accomplished using Vectastain Standard ABC Kit (Vector), with NiDAB (Sigma-Aldrich) as a substrate. After rinsing in PBS, sections

were dehydrated in an alcohol series, cleared with xylene and mounted with Permount (Fisher Scientific). For fluorescence immunohistochemistry, following both antigen retrieval steps, the slides were exposed to primary antibodies for rabbit-anti-pH3 (Millipore, 1:1000) and rat anti-BrdU (Accurate; dilution 1:1000) overnight at 4°C. After washing in PBS, the slides were incubated for 1 h in secondary donkey anti-rabbit Alexa Fluor 555 (Invitrogen, 1:1000) and donkey anti-rat Alexa Fluor 488 (Invitrogen, 1:1000). The sections were washed in PBS and mounted using Vectashield mounting medium (Vector).

Microscopy and Analysis

Light microscopy images were taken with a Zeiss Axioskop microscope equipped with Q Color 5 Olympus camera, 20X objective, and Q Capture Images software. Confocal microscopy was performed with a Zeiss LSM 710 using the Observer Z1 inverted microscope. Using Zen software, images were taken with a 20X objective. To minimize crosstalk between channels in multicolored tissue, sequential image acquisition was performed. The absolute number of labeled cells was quantified in the entire dorsal telencephalic, preoptic area, habenular, hypothalamic and cerebellar neurogenic niches (according to Grandel et al., 2006). To account for inter-individual difference in brain size, the data were adjusted for brain volume, which was estimated based on images of individual brain sections (ImageJ) and using the Cavalieri principle (Prakash et al., 1994). Statistical significance for mean values was assessed with an unpaired Student's t test, with significance level of $p < 0.05$.

Real-time quantitative RT-PCR (qPCR)

At each time point, groups of fish were submerged in liquid nitrogen. Brains were dissected out on dry ice and stored at -80 °C, until analysis. RNA extraction from brain tissue was conducted using QIAzol Lysis Reagent (Qiagen) before RNA isolation using the RNeasy Qiagen kit. The quantity and quality of RNA was determined spectrophotometrically, at 260/280 nm. The same amount of RNA from each sample was converted into cDNA using the High-Capacity cDNA Archive Kit (Applied Biosystems) according to the manufacturer's instruction. Q-PCR was performed using a TaqMan® Universal PCR Master Mix or SYBR Green PCR Master Mix and ABI Prism 7300 Real Time PCR System (ABI). The TaqMan® probes and SYBR Green primers were based on the following sequences: Cyclin D1: (Forward, 5'-GTC GCG ACG TGG ATG C-3', Reverse, 5'-CCA GGT AGT TCA TAG CCA AAG GAA A-3', {TCG AGG TCT GTG AAG AGC AG}); Cyclin E: (Forward, 5'-AGC GCT TTA GAT TCA AGA ACC TCT T-3', Reverse, 5'-GCT CCT TGT TCA GCA TCT TTA TCC A-3', {CCA GAC CCC TGT TTA GC}); Cyclin A2: (Forward, 5'-TGG AGA ACA ACC AGA GGA GAC A-3', Reverse, 5'-GCA TTT TCT TCA GGT TTA CAC GCA AT-3', {CCA GAC CCC TGT TTA GC}); Cyclin B2 (Forward, 5'-GCG AAC TGT CTA ATC TTT CCC ACA A-3', Reverse, 5'-CGG CCA GTG GGT TTT ACA C-3', {CAG TTC AGA CAA AGA AGG TT}); *Bmal1*: (Forward: 5'-CAG AGC TTC GCC ACA AAC C-3', Reverse, 5'-CTG TGA TCA ATG CAT GTC CTT TCA-3', {CTCGATGTGAGGATCTG}); *Clock1*: (Forward: 5'-CAT CCT ACA GAA GAG CAT CGA CTT-3', Reverse 5'-GAT TTC ACT CGA CTC CGA CTG T-3', {AAG CAC AAA GAA ATT G}); *Per1*: (Forward:

5'ATT CCG CCT AAC CCC GTA TGT GAC C-3', Reverse: 5'GTG TGC CGC GTA GTG AAA ATC CTC TTG T-3'); *P20*: (Forward: 5'-GGT CCG TGT GGA CTT GAT TT-3', Reverse: 5'-CCT CTT CAA CAG CCC ATG AT-3'); *Wee1*: (Forward: 5'-CAT CGC CAC GGA AAG TC-3', Reverse: 5'-TGG GGG GTA TCA AAA AGA C-3'); *P21*: (Forward: 5'-CCG CAT GAA GTG GAG AAA AC-3', Reverse: 5'-ACG CTT CTT GGC TTG GTA GA-3'). Gene expression was normalized for two house-keeping genes: *β -actin* and *EF1a* expression level for each individual fish sample: *β -actin*: (Forward, 5'-GCT GTT TTC CCC TCC ATT GTT G-3', Reverse, 5'-TTT CTG TCC CAT GCC AAC CA-3'; {CCC AGA CAT CAG GGA GTG}); *EF1a*: (Forward, 5'-GCA CGG TGA CAA CAT GCT-3', Reverse, 5'-TCC TTG CGC TCA ATC TTC CAT-3'; {ACC AGC CCA TGT TTG AG}). Relative mRNA expression level was calculated using the standard comparative delta-Ct method.

Statistical modeling of circadian kinetics of S-phase entry and S length

For a given neurogenic niche, the distribution of phase angles of S-phase initiation within a given day $G(t)$ was modeled as a Gaussian function with a mean μ , dispersion σ , multiplied by a rate scaling constant r . The length of the S-phase was modeled as a constant value of ϵ . The resulting number of BrdU labeled cells $n(t)$, measured at a time t_0 was then modeled as a sum of all cells that were within the S-phase during BrdU labeling. To account for the circadian periodicity of the signal, the contributions of cells originating from different days (labeled by index k) are summed up:

$$(t_0) = \sum_{k=-\infty}^{\infty} \left[\int_{t_0-\alpha+\beta-\varepsilon}^{t_0-\beta} G(t + k\tau) dt \right]$$

where α is the length of the BrdU labeling (4 h), β is the minimal amount of time a replicating cell needs to overlap with the BrdU pulse to be reliably labeled (1 h), and τ is the length of the circadian cycle (24 h). Given the numbers of the BrdU labeled cells observed at different time points, the posterior distributions of the remaining parameters (μ , σ , r , ε) were estimated following a Bayesian approach, using No-U-Turn sampler (Hoffman and Gelman, 2011), as implemented by the Rstan package. Prior distribution for μ was taken to be $N(12h, 24h)$. σ prior was taken to be a Gamma distribution with shape of 2.5 and rate of 0.5. Prior for r was taken to be Gamma distribution with shape of 3 and rate of $3/p$, where p is the maximum observed number of BrdU labeled cells. The prior distribution for ε was taken to be a broad beta distribution with parameters 1.5 and 1.5, rescaled to the 24 h time period. For computational efficiency only three adjacent days were taken into account (i.e., only values of k equal to -1, 0, and 1 were considered), as contribution of more distant days was found to be negligible. To estimate joint posterior distributions, a total of 5000 sampling iterations were performed.

Chapter 2.4: Results

Daytime S-phase progression is common to all neurogenic niches, with niche-specific distribution of the phase angle of S initiation and S length.

To characterize circadian variation in the number of cells undergoing S-phase in adult brain under entrained light-dark conditions, groups of zebrafish were exposed to BrdU for 4 h, at multiple time points throughout the 24-h cycle. Further evaluation of the number of BrdU-positive cells was conducted in five neurogenic niches, including the dorsal telencephalon (DT), parvocellular preoptic area (PPa), habenula (Hb), hypothalamus (Hyp) and cerebellum (Cer). Out of 16 neurogenic niches documented in adult zebrafish and distributed along the whole rostro-caudal brain axis, we chose these five based on earlier studies demonstrating either their high level of homology to mammalian structures (Cer, Hb, Hyp, PPa) or homology to specific areas of adult neurogenesis in mammals (e.g., DT being homologous to hippocampus) (Zupanc et al., 2005; Kaslin et al., 2009; Cheng et al, 2014). Moreover, Cer and DT represent some of the most active proliferating zones in adult zebrafish brain, with different stem cell types present (Kaslin et al., 2013; Than-Trong and Bally-Cuif, 2015).

All five niches revealed daily variation in the number of cells undergoing DNA replication (Fig. 6). The number of BrdU-labeled cells, total and at peak, was highest in Cer (1183 ± 178.7 , peak), with minimal numbers observed in Hb (30 ± 8.3 , peak), reflecting the difference in the overall size and proliferative capacity of these niches (Fig.

6D, E). Common to all niches was a robust increase in the number of S-phase cells by mid-day (ZT7), with a peak in the evening and early night hours (ZT11-ZT19), and a trough at the end of the night (ZT23) (Fig. 6A-H). Additional experiments with more frequent sampling rate over shorter intervals demonstrated similar number of BrdU-positive cells at trough, between ZT23 and ZT1 (data not shown). Such non-zero values at the troughs indicate that some fraction of dividing cells might be uncoupled from the circadian cycle. However, the magnitude of circadian oscillation significantly exceeded this background level in all examined structures (Fig. 6I).

To quantify the circadian kinetics of CDC, we constructed a statistical model. From the observed BrdU patterns within each neurogenic niche studied, we modeled the distribution of phase angles of S-phase initiation within a given day as a Gaussian function $G(t)$ with a mean μ , dispersion σ , and the length of the S-phase as a constant ϵ (see Methods). Following Bayesian approach we evaluated the posterior distributions of these parameters based on the likelihood of attaining the observed cumulative counts of BrdU-labeled cells at different time points throughout the cycle (Fig. 7A-D). We found that the mean phase angle of entry into S-phase (μ , measured relative to the onset of environmental illumination ZT0) occurs during the first quarter of the day, but varies between different niches (Fig. 7E), with PPa showing the latest onset (maximum likelihood estimate 5.8 h), and Cer the earliest (3.1 h). Similarly, neurogenic niches showed significant variation in the degree of synchrony of the S-phase entry by individual cells, as captured by the phase angle dispersion σ (Fig. 7F), with Hb showing

significantly higher σ (lower synchrony) than all other niches ($p=0.035$). Finally, the average length of the S-phase (ϵ) also showed notable variation between the niches (Fig. 7F), with ϵ predicted for DT (15.2 h) and Cer (16.1 h) being significantly longer than that for PPa (10.7 h) or Hb (10.6 h) ($p=0.011$).

These results indicate that while circadian dependency of cell proliferation rates is pronounced across all of the examined niches, the prevalent timing of S-phase initiation, the degree of synchronization between the cells, and the length of the S-phase itself varies significantly between the neurogenic niches.

Nighttime progression of cells through G2/M phase in adult neurogenic niches.

We used the phosphorylation of histone H3 (pH3) at serine 10, a relatively short-lived marker present specifically in late G2 and early M phases (Hendzel et al., 1997), to determine the daily pattern of G2/M in individual neurogenic niches. Daily variation in pH3-positive cells was first evaluated at 4-6 h intervals, suggesting predominantly nighttime expression (data not shown). This was further confirmed using higher temporal resolution, with pH3 being documented at 2-h intervals (Fig. 8A, B). The increase in pH3 labeling was observed throughout the night, with a peak at ZT23. This was consistent with the trough in BrdU-labeled cells at that time (Fig. 1D), confirming that by the end of the night the majority of dividing cells completed the S/G2 transition, proceeding to mitosis.

While all the fish were exposed to BrdU for 2 h before sample collection, in some cells only pH3 could be documented, while others were co-labeled for pH3 and BrdU (Fig. 8A, B). For cells to express only pH3, they had to exit S-phase at least 2 h before documented expression of the G2/M marker (Fig. 8A). In contrast, those co-labeled for BrdU and pH3 had to accomplish both S and at least part of G2 within the 2-h interval of BrdU exposure, indicating that the length of their G2 until the time of pH3 expression was substantially shorter than 2 h (Fig. 8B). The number of such double-labeled cells declined overnight. Together, this suggested potential difference in G2 length in cells within the same neurogenic niche, and increase in G2 length overnight.

Entrained and intrinsic rhythms of mRNA expression for cell cycle regulators and core clock genes in adult zebrafish brain.

To further explore the timing of the cell cycle phase transitions in adult brain and their correlation with the core circadian clock machinery, the mRNA expression patterns for critical cell cycle and circadian clock regulators were assessed using qPCR (Fig. 9). We analyzed expression of cyclins involved in G1/S transition (D1 and E), S progression (A2) and G2/M (B2) (for review, Stacey, 2003). We also documented expression patterns for cyclin-dependent kinase inhibitor p20 (CDK-p20) that limits transition between the G1 and S phases (Laranjeiro et al., 2013). Two genes representing the positive and negative parts of the feedback loop of the clock, respectively, were *Clock1* and *Per1* (Fig. 10).

The expression of cyclin A2 was increased throughout late day and into the night hours (Fig. 9A). The expression of *p20* peaked at approximately the same time, with acrophase around ZT 11-19 (Fig. 9B), preceding robust decline in the number of S-phase cells (Fig. 1). The expression of cyclin B2 also peaked at day-night transition (Fig. 9C). Thus, late day and early night upregulation of mRNA abundance involved genes critical for the middle and end of the cell cycle, i.e., S-phase progression and G2/M. In contrast, the expression of cyclins involved in the early phases of the cell cycle occurred in the morning. The expression of cyclin D1, which controls progression through late G1 phase, was increased by the end of night and peaked in the early morning hours, at ZT3 (Fig. 9D). The expression of cyclin E1, involved in G1/S checkpoint transition (Ohtsubo et al., 1995), typically surged close to the cyclin D peak time, but its pattern was highly inconsistent between independent experiments, in contrast to other cyclins (data not shown). We assessed two other cell cycle regulators in the zebrafish brain, *Wee1* and *p21*, known to be under clock control in other tissues (Matsuo et al., 2003; Grechez-Cassiau et al., 2008; Laranjeiro et al., 2013), but those displayed very low mRNA abundance in zebrafish brain, with no indication of daily variation present (data not shown). Similar to what we have reported earlier (Zhdanova, et al., 2008), the *Clock1* transcript peaked at the end of the day (ZT11-15), reaching trough by the end of the night, ZT23 (Fig. 10A), while *Per1* mRNA expression in zebrafish brain reached its peak late at night, ZT23 (Fig. 10B).

To determine whether the observed daily rhythms of expression for the principal cell cycle regulators reflect intrinsic circadian oscillations, animals were studied in constant darkness (DD). The mean circadian variation for the core clock genes, while studied in LD, was 135 fold for *Per1* and 3.6 fold for *Clock1*. Following 48-h in DD, the acrophase for the clock genes remained similar to that in LD, while the magnitude of variation for *Clock1* and *Per1* was significantly reduced (Fig. 10 A, B). This was anticipated based on earlier reports in diverse species of entrained rhythms of activity or gene expression having significantly higher amplitude when compared to the intrinsic rhythms (Tamai et al., 2005; Huang et al., 2011; Hughes and Piggins, 2014; Ramkisoensing and Meijer, 2015). Moreover, a desynchronization between the fish with different intrinsic circadian period can be expected in the absence of entrainment to light, further reducing mean group amplitude. No significant circadian variation in cyclin patterns could be documented in DD (data not shown), potentially due to their low baseline circadian variation in LD (2-3 fold; Fig. 9), comparable to the degree of reduction in clock gene expression between entrained and constant conditions. In contrast, a higher magnitude of daily variation in *p20* transcript expression with a peak of 10.4 ± 2.0 fold, though also substantially decreased, retained significant intrinsic circadian variation in DD (Fig. 10 C). Together, this suggested presence of intrinsic CDC rhythm, with entrainment to light enhancing circadian oscillations in adult neurogenesis.

The G1/S transition in the morning hours enables progression of the cell cycle over the following 24-h period.

Based on a combination of the observed daily expression patterns for cyclins and *p20*, daytime increase in S-phase progression (as per BrdU), and late G2/M-phase peaking by the end of the night (as per pH3), we hypothesized that upregulation of cyclin D expression throughout the morning reflects the timing of the G1/S transition for the cells undergoing division over the next 24-h period. We thus predicted that interfering with cyclin D effects at the end of Night 1 and in the morning hours would reduce BrdU labeling throughout the Day and expression of cyclins A2 and B2 on Night 2, reflecting a diminished population of cells progressing through S and G2/M earlier in the day.

To test this hypothesis, we used PD 0332991, a highly selective inhibitor of the cyclin D kinases, CDK4 and CDK6, which blocks retinoblastoma protein (pRb) phosphorylation, inducing G1 arrest independent of the presence of functional D1 cyclins in the cell (Fry et al., 2004). The inhibitor was administered starting the end of Night 1 (ZT22), when mRNA expression for cyclin D1 surges. Fish were then continuously exposed to the drug until ZT6', to cover the period of increased D1 transcript expression and rapid increase in the number of BrdU positive cells, requiring cyclin-activated D1 kinases to move cells toward the G1/S checkpoint. Such alteration in cyclin D1-regulated pathway did not interfere with the expression levels for the cyclins on Night 1 or early morning, suggesting lack of acute effect on cyclin production (data not shown). Importantly and consistent with our hypothesis, the treatment led to the reduced expression of cyclins A2 and B2 on Night 2, with expected lack of changes in cyclin D1 expression (Fig. 11A).

This was further supported by significantly reduced BrdU labeling on Night 2 (Fig. 11B). Together, this indicated that G1 arrest on Night 1 and in the morning alters transition of cells through S-phase later that day and their progression toward mitosis on Night 2, thus supporting our hypothesis.

Chapter 2.5: Discussion

This study establishes that, in diverse adult neurogenic niches of a diurnal vertebrate, cell cycle progression displays robust entrained circadian rhythm. Starting in the morning, cells gradually transit from G1 to S phase of the cell cycle. This process spreads over several hours, with a peak number of cells undergoing S-phase observed close to the time of light-dark transition. Thereafter, new cells are prevented from entering S phase, at least in part due to activation of the cyclin-dependent kinase inhibitor p20 around that time. This is followed by cells gradually exiting S and transiting into G2/M phase throughout the night, with peak in G2/M observed by the end of the night (Fig. 12).

Under stable environmental conditions in the presence of environmental light-dark cycle, CDC is coupled to the circadian clock rhythm in zebrafish brain (Fig. 12). The peak in S-phase corresponds to the activation of the positive arm of the circadian loop, as per expression pattern for *Clock1* mRNA. The CDC is completed at the time of upregulation of *Per1* mRNA expression, toward late night and early morning hours. Preservation of circadian variation in transcript levels for a prominent CDC regulator, *p20* (Fig. 10C), in

the absence of entraining environmental photoperiod also suggests that the circadian pattern of CDC in zebrafish brain can be intrinsic in nature.

Several earlier reports implied that phase relationship between the clock and CDC in diverse tissues can be largely conserved between species and preserved in vitro. In cultured human fibroblasts, in spite of a much shorter than 24-h period, the peak of expression for Rev-erb transcript that, similar to *Per1*, reflects a negative feedback loop of the clock is coupled to the G1/S transition (Feillet et al, 2015). Also consistent with our results, the *p20* transcript levels peak at day-night transition in zebrafish cell lines, embryos and adult brain tissue (Laranjeiro et al., 2013). Interestingly, in night-active mice, cell proliferation in the subgranular zone (SGZ) of adult hippocampus is also increased at night (Bouchard-Cannon et al, 2013). However, it shows substantial delay in the time of initiation, relative to the typical timing of S-phase entry we find in day-active zebrafish, and higher background levels. While our data demonstrate a consistent pattern of circadian variation in CDC progression across all examined zebrafish neurogenic niches, there is lack of such consistency in mice. The surge in the number of M-phase cells in SGZ at nighttime is in contrast with lack of such circadian variation in the second neurogenic niche in mice, the subventricular zone (SVZ) of the forebrain, and no significant variation in S-phase observed in either region in these animals (Tamai et al., 2008). Further in-depth investigation into the differences in circadian regulation of neurogenesis between diurnal and nocturnal species could be of principal importance for

translational research, in view of the inverse relationship between the central clock and downstream clock-controlled processes in these animals.

We conducted comparison between CDC patterns documented in individual neurogenic niches of zebrafish brain. Since BrdU labeling provides cumulative tallies of the number of cells undergoing divisions, we relied on statistical modeling to estimate the rate at which the cells in different neurogenic niches enter the S phase throughout the 24-hr period. The results confirm that circadian dependency of cell proliferation rates is pronounced across all of the examined niches. They also reveal significant differences between the neurogenic niches, with respect to the magnitude of circadian oscillation, prevalent timing of S-phase initiation (phase angle), the degree of synchronization between the cells, and the extent of the S-phase length. For example, the cerebellar niche (Cer), the largest in zebrafish brain, has high magnitude of circadian oscillation, reflecting relatively low numbers of S-phase cells at day/night transition and thus fewer cells that are not following the prevalent temporal pattern of cell cycle progression. Cer is also the niche with the most advanced phase angle of S entry, corresponding to a more synchronized G1/S phase transition among dividing cells. In contrast, another relatively large neurogenic niche located in dorsal telencephalon (DT) has low magnitude of daily oscillation, with high number of cells not following typical circadian pattern of CDC. The DT also has late S-phase onset and low synchronization between cells. This is in spite of both Cer and DT niches having similar mean S-phase length, substantially longer than in other niches studied.

These results raise intriguing questions of whether inter-niche differences in cell cycle progression might reflect different strength of central circadian regulation in these brain regions. Presence of local niche-specific factors, related to diverse physiological functions and neuronal connections of these brain regions, might also synchronize cells in their transition through CDC in a niche-dependent way. Similarly, difference in the mean length of S-phase suggested by our model might reflect homogenous populations of niche-specific cells or different proportion of cells with longer and shorter S-phase length in each niche, as shown in some earlier studies (Brandt et al., 2012; Feillet et al, 2015). Moreover, our data on the presence or absence of co-expression of S- and G2/M phase markers (BrdU and pH3) over consecutive 2-h intervals also suggest that the length of G2 can vary between cells, increasing overnight.

It remains to be seen which cell qualities could reflect different CDC kinetics. For example, the timing at which dividing cells transit G1/S and the length of their S or G2 phases might differ between actively self-renewing multipotent neural stem cells (NSCs) and amplifying neural progenitor cells (NPCs) with limited number of divisions (Grandel et al, 2006). The known NSCs heterogeneity and plasticity (Kaslin et al, 2013; Than-Trong and Bally-Cuif, 2014; Barbosa and Ninkovic, 2016) might also contribute to the variation in circadian patterns of CDC progression. It will be important to determine whether adhering to a well-synchronized circadian pattern of cell division might help in maintaining a healthy pool of specific NSCs with broad potential or NPCs with restricted potential within adult neurogenic niches.

The timing of G1/S transition, the degree of synchrony between the cells of the same niche in entering S, or the length of S and G2/M phases might also correlate with the survival, migration or neuronal fate of new cells in adult brain. This first study on the circadian variation of CDC in neurogenic niches of adult zebrafish brain did not aim at addressing these issues. However, further studies, requiring extensive pulse-chase investigation initiated at different times of day, with cells being followed by several neuronal and glial markers over prolonged periods of time, could establish the extent to which the timing of cell progression through S or M phases might affect cell fate.

Previously, it was reported that the vast majority of dividing cells in adult zebrafish brain give rise to neurons, with only a minority of cells differentiating into glia (Grandel et al, 2006; Kaslin et al, 2009; Kaslin et al, 2013). This and only a small number of cells replicating outside the typical circadian cycle, as per the background S-phase cells we observed late at night and early in the morning, suggest that the circadian pattern of CDC in neurogenic niches reported here is likely to reflect circadian regulation of adult neurogenesis in zebrafish brain.

To summarize, the robust circadian variation in the transition of cells through CDC within adult neurogenic niches demonstrated here using a diurnal vertebrate model suggests that a similar phenomenon might be present in the brain of day-active humans. Understanding the circadian phase of entrainment of CDC phases to environmental and endogenous factors, or time-dependent response of adult neurogenesis to pharmacological interventions, could be of major clinical significance. The timing of

such interventions, aiming at either enhancing neurogenesis in brain trauma or neurodegenerative disorder patients, or preserving normal neurogenesis during cancer cytostatic therapies, could be critical. Considering the advantages of the model, the search for local niche-specific factors that can modulate daily CDC progression in zebrafish brain may lead to novel prophylactic and treatment strategies.

Chapter 2.6: Figures

Figure 6. Daily pattern of S-phase of the cell division cycle in five neurogenic niches of adult zebrafish brain.

A. Schematic of five neurogenic niches of adult zebrafish brain, with box identifying the areas presented in B and C. **B, C.** Representative images of daily variation in the number of BrdU+ cells in each niche, at daily trough (B) and peak (C), as presented in **D**. Scale bar: 50 μ m. **D.** Number of BrdU+ cells undergoing S-phase within each neurogenic niche, documented at 4h intervals over a 24-h period. N=4-6 fish per time point, mean $\pm$ SEM, *P<0.05 relative to the trough in BrdU+ cells for specific niche. Grey background - night, 14:10 LD cycle. Samples collected at specified zeitgeber time (ZT), following a 4-h in vivo exposure to BrdU. ZT0 = lights-on time. **E.** Schematic of zebrafish brain identifying coronal planes at which the brain was cut to evaluate BrdU+ cells presented in B-D. **F.** Range of circadian oscillation, the ratio of the number of BrdU-labeled cells at peak and trough in each niche (folds).

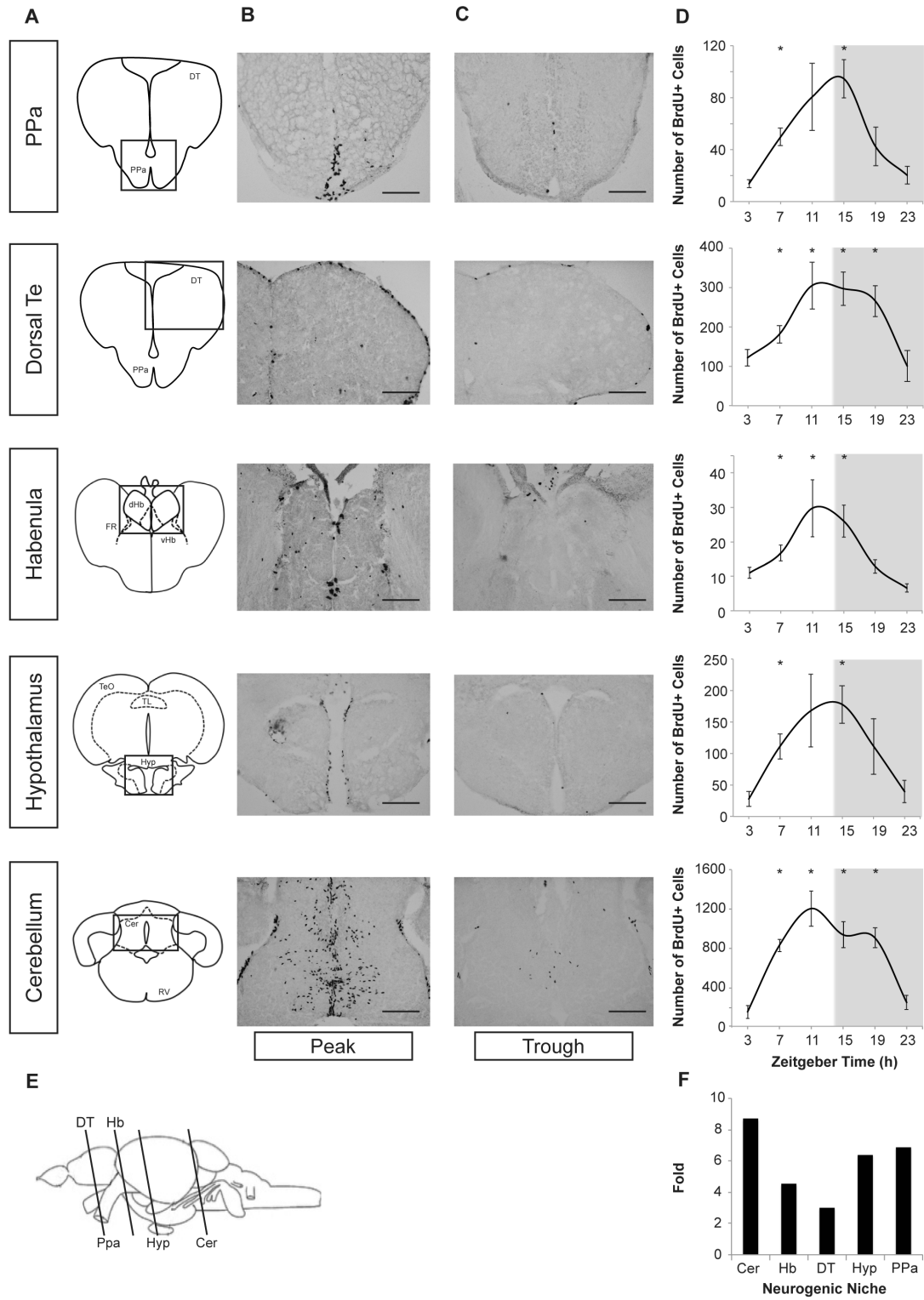


Figure 7. Statistical modeling of circadian kinetics of S-phase progression in diverse neurogenic niches of adult zebrafish brain.

A. Boxplots show the number of BrdU+ cells in DT niche, observed at different time points throughout the day. The values for each individual fish are shown as red dots. The black curves show the overall fit of the statistical model (see Methods) for the observed number of BrdU+ cells. The rate of S-phase entry predicted by the model is shown by blue curves. For both curves, the solid line shows the maximum likelihood model predictions, and thin lines around it show predictions sampled from the posterior ensemble of the likely model parameter estimates. **B.** Marginal posterior distributions of the individual model parameters for the DT niche, including mean (μ) and dispersion (σ) of the S-phase entry rate function $G(t)$, and the length of the S-phase (ϵ). The shaded region under the curve marks the 95% credible interval. **C, D.** Fit illustration and posterior distributions for the PPa niche. **E, F, G.** Marginal posterior distributions of the μ , σ , and ϵ parameters are shown for all of the examined neurogenic niches.

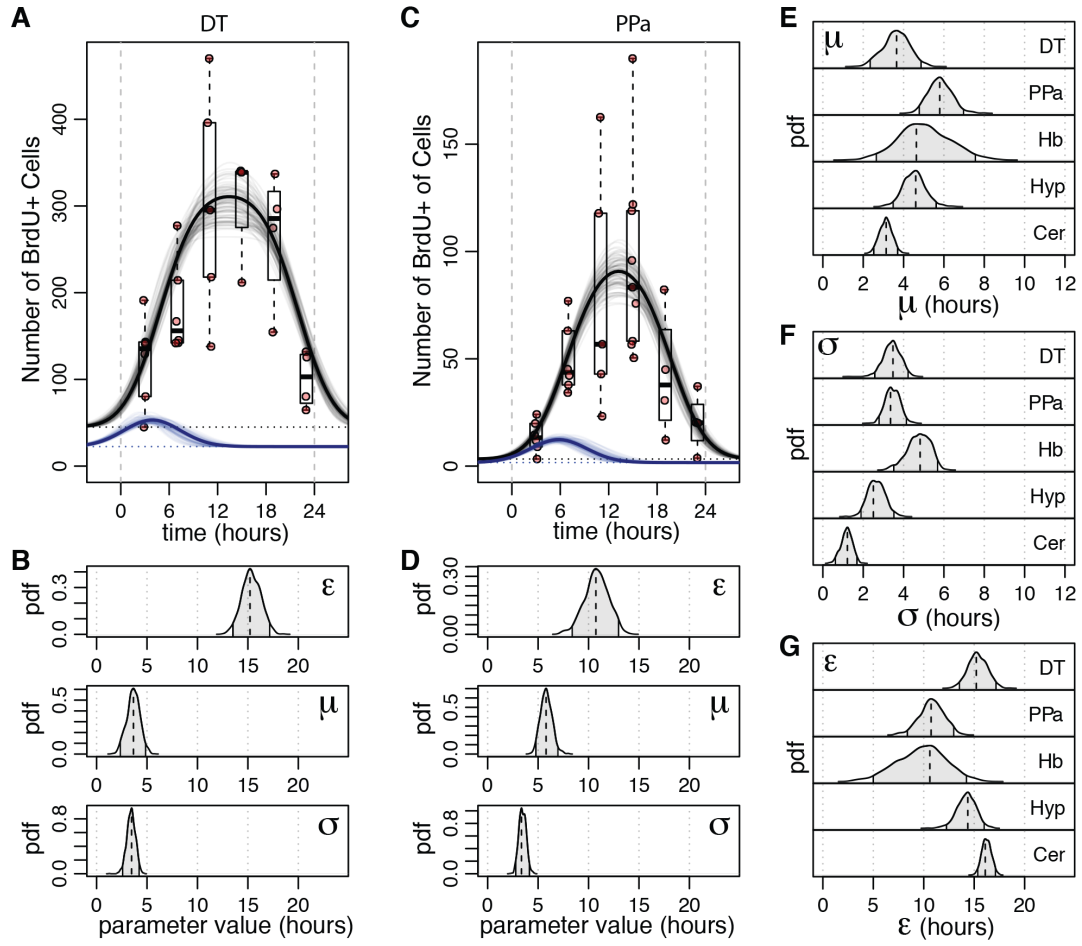


Figure 8. Nighttime transition of dividing cells through G2/M phases of cell cycle in the cerebellum of adult zebrafish.

A-C. Number of cells expressing pH3, a late G2 and early M phases marker, and BrdU, a marker for S-phase. Cells positive for pH3-only increase throughout the night (**A**); cells with co-localized BrdU/pH3 label (**B**) decline throughout the night. N=3-6 fish per time point, mean $\pm$ SEM, *P<0.05 relative to the trough in labeled cells (pH3 only, ZT11; BrdU/pH3, ZT23). Grey background - night, 14:10 LD cycle. Fish collected at specified zeitgeber time (ZT), following a 2h in vivo exposure to BrdU. ZT14 = Lights-off time. Representative confocal image (**C**): cells labeled for pH3-only (red), BrdU-only (green), and BrdU/pH3 merge (yellow). Scale bar: 20 μ m.

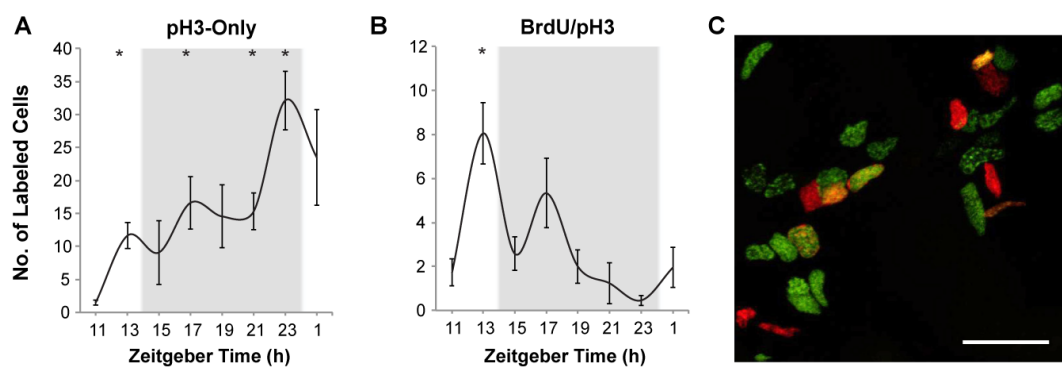


Figure 9. Daily cycle of mRNA expression for cell cycle regulators in adult zebrafish brain.

A-D. Transcript expression for Cyclin A2 (**A**), *p20* (**B**), Cyclin B2 (**C**), Cyclin D1 (**D**).

* $P < 0.05$ relative to the time of trough for mRNA abundance for each gene (ZT3' for Cyclin A, *p20*, Cyclin B2, and ZT19 for Cyclin D1). N=5-6 fish per time point, mean $\pm$ SEM. Grey background - night, 14:10 LD cycle. Zeitgeber time of sample collection, ZT0=Lights-on time.

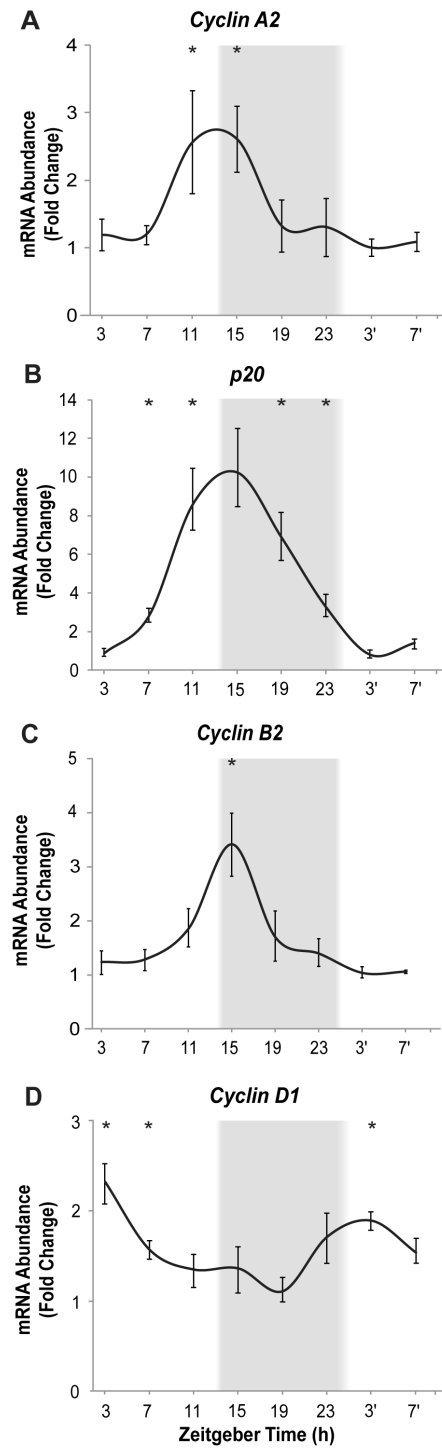


Figure 10. Intrinsic and entrained circadian variation in mRNA abundance for p20 and core clock genes.

A-C. Circadian patterns of mRNA abundance for *Clock1* (*A*), *Per1* (*B*), and *p20* (*C*), under LD (solid line) and DD (dashed line) conditions. N=5-6 fish/time point, mean±SEM. Grey background - night, 14:10 LD cycle. Zeitgeber time of sample collection, ZT0 = Lights-on time. Intrinsic circadian rhythm in DD, *P<0.05, relative to the time of trough for mRNA abundance for each gene in DD (ZT23 for *Clock1*, ZT11 for *Per1*, ZT3' for *p20*). Reduction in mRNA abundance at peak, DD vs. LD, #P<0.05 (ZT15 for *Clock1* and *p20*, ZT23 for *Per1*).

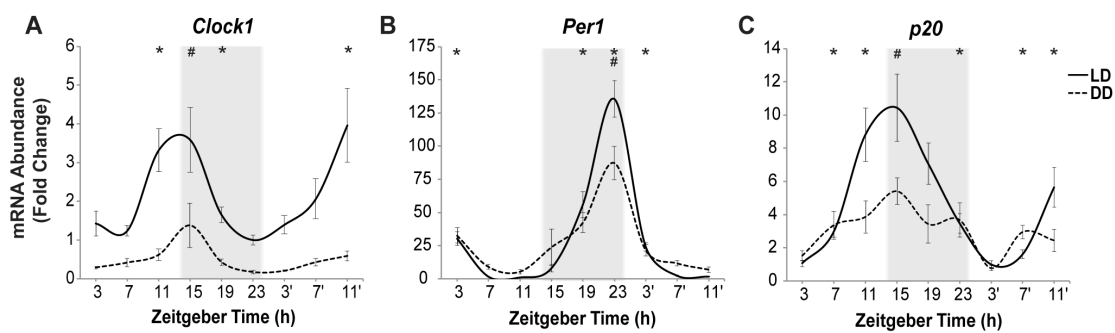


Figure 11. Interference with cyclin D function on Night 1 inhibits S- and G2/M-phase progression over the following Day and Night 2.

A. Administration of PD 0332991, a CDK4/6 inhibitor, at ZT 22 of Night 1 leads to a reduction in the expression of cyclins A2 and B2 on Night 2 (ZT19' & 23', combined).

B. Reduction in the number of BrdU+ cells during the Day (ZT11') and Night 2 (ZT15'), following a 4-hour exposure to BrdU (immersion). N=5-6 fish/time point, mean±SEM,

*P<0.05 relative to control. ZT0 = Lights-on time.

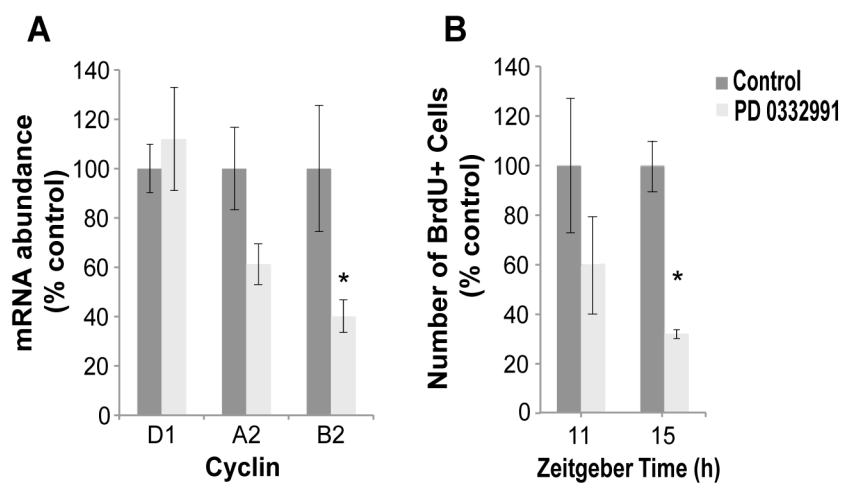
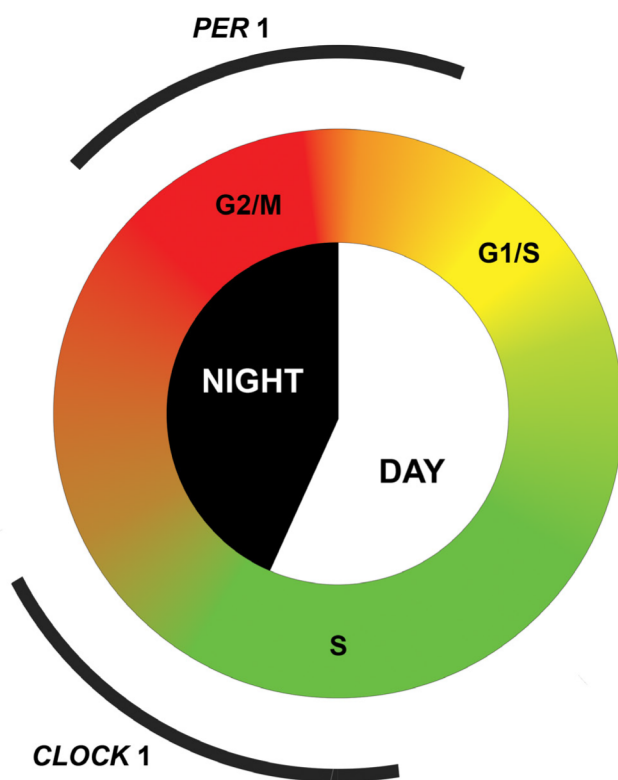


Figure 12. Schematic of the typical cell cycle progression in adult brain of a diurnal vertebrate, zebrafish, maintained under regular light-dark conditions.

At early morning hours of Night 1, upregulation of cyclin D1 expression initiates gradual G1/S transition in the population of cycling neuronal stem and progenitor cells.

Throughout the Day, S-phase initiation and DNA replication continues, with gradual surge in cyclin A2 expression and the number of S-phase cells peaking late in the day.

Surge in *p20* expression is initiated by the end of the Day, limiting further S phase initiation. These CDC events are coupled to the activation of the positive feedback loop of the circadian clock, as per *Clock1* transcript levels. Cyclin B2 expression is upregulated thereafter, promoting late G2 and M phases of the cell cycle throughout Night 2, with the cell cycle culminating by the end of the night. The end of the cycle and the preparation for the next G1/S transition is coupled to the initiation of the negative feedback loop mechanisms of the clock, coinciding with upregulation of *Per1* transcription at the end of the night.



CHAPTER THREE: THE KINETICS OF CELL DIVISION, TURNOVER AND MIGRATION WITHIN A NEUROGENIC NICHE OF A DIURNAL VERTEBRATE.

Chapter 3.1 Abstract

This Chapter is devoted to our investigation into the kinetics of cells composing a neurogenic niche in young healthy adult zebrafish. The events occurring in the cerebellar niche over several consecutive days revealed orderly patterns of cell division, maintenance and turnover of principal cells composing the niche. Studies described here, both experimental and involving mathematical modeling, determined a consistent number of neural stem cells (NSCs) dividing daily, the predominant division mode for transient amplifying cells, the neural progenitors (NPCs), and the pace of migration and survival of their progeny.

Chapter 3.2 Introduction

New neurons are constantly produced and turned over throughout life in the adult vertebrate brain. The discovery that these neurons differentiate from adult neural stem cells (NSCs) that are localized to discrete neurogenic niches raised the possibility of manipulating these NSCs for regenerative therapy of neurodegenerative diseases or brain trauma. NSCs are typically characterized as cells with relatively rare cell divisions but an extensive if not unlimited ability for self-renewal and multilineage potential. NSCs generate transient neural progenitor cells (NPCs), which are limited in their proliferation potential and eventually differentiate into neuroblasts/glioblasts (NB/GB), giving rise to

new neurons and glial cells. The kinetics of NSC and their progeny remain poorly understood.

As described in detail in Chapter 1 (Fig. 3 and 4), there are multiple modes of division for NSCs and NPCs. The balance of these modes of division leads to different scenarios within a niche, and can result in a loss of NSCs, low or high production of NPCs or their progeny, NB/GB. Moreover, there is a need for the progeny of NPCs or, in case of a direct conversion also the progeny of NSCs, to migrate out of the niche, providing space for new generation of cells. Together, this complex kinetics of diverse cells requires orderly turnover of transient cells and their products for the neurogenic niche to stay healthy and productive. Understanding these processes would allow us to determine their vulnerability to diverse pathological conditions and a way to potentially treat those conditions.

Zebrafish have emerged as a powerful model to study adult neurogenesis due to robust proliferative capacity of their brain, with NSCs localized to 16 discrete niches along the entire rostro-caudal axis (Grandel et al., 2006). Similar to mammals, these niches produce neurons and glial cells specific to individual neurogenic niches (Zupanc et al., 2005).

A pulse-chase approach using two complementary S-phase markers and mathematical modeling allowed us to conduct detailed characterization of the behavior of cells within the cerebellar niche of the adult zebrafish, the niche with the highest proliferative capacity in the zebrafish brain. The results of this investigation are presented here.

Chapter 3.3: Materials and Methods

Animals

Adult male zebrafish (*Danio rerio*, wild-type AB strain), 12 $\pm$ 1 months old, were maintained on a 14 h light:10 h dark (14:10 LD) cycle, at 28°C, in 3L tanks of a multi-tank system (Aquaneering), as per standard practices (Whitmore et al., 2000). Larval diet consisted of live food of *paramecium* and Type L saltwater rotifers (*Brachionus plicatilis*). Regular feeding schedule for mature fish consisted of twice a day administration at zeitgeber times (ZT) 1 and 8, (ZT0=lights on time) of pellets Gemma 300 (1.7% of body weight), with supplement live feed *Artemia salina* nauplii/metanauplii (brine shrimp). All animal procedures were performed in accordance with Institutional Animal Care and Use Committee at Boston University School of Medicine.

5-bromo-2'-deoxyuridine (BrdU) and 5-ethynyl-2'-deoxyuridine (EdU) Pulse-Chase

Treatment

On Day 0, three groups of fish (1-year old) were exposed to BrdU in tank water for a 2-h period (ZT9-11), corresponding to peak in the number of cells in S-phase (Fig. 6). Following the 2-h period, the tanks were then washed out. On each of 3 consecutive days, one group of fish was injected (i.p.) with EdU (50 μ g/g) and sacrificed 2h later, at ZT11, i.e., 24-72h after BrdU exposure (Fig. 13).

Immunohistochemistry

Fish were euthanized with an overdose of MS222 (200 mg/l) and heads were fixed overnight in 4% paraformaldehyde in phosphate buffered saline (PBS) at 4°C. Brains were then dissected out, cryoprotected in 30% sucrose/PBS, placed in embedding solution (O.C.T) and stored at -80°C, until cut. Coronal 20 µm sections were prepared using a cryostat (Microm HM505E) and placed onto Fisherbrand Superfrost Plus slides (Fisher), then stored at -80°C, until processed. The sections were washed in 0.1 M PBS and antigen retrieval was performed using both heat, incubated in 50% formamide / 50% 2X SSC at 65°C for 2 h, and acid, 2 M HCl at 37°C for 30 min and 0.1 M boric acid (pH 8.5) for 10 min at room temperature. Following a PBS wash, the slides were incubated overnight in mouse anti-BrdU antibody directly conjugated to AlexaFluor 555 (Life Technologies, 1:20; in 0.1 M KPBS + 0.4% Triton-X100) at 4°C. After washing in PBS, sections were incubated in Click-iT® reaction cocktail for 30 min from the Click-iT® EdU Alexa Fluor® 488 Flow Cytometry Assay (Invitrogen). After rinsing in PBS, slides were mounted using Vectashield mounting medium (Vector).

Microscopy and Analysis

Confocal microscopy was performed with a Zeiss LSM 710 using the Observer Z1 inverted microscope. Using Zen software, images were taken with a 20X objective. To minimize crosstalk between channels in multicolored tissue, sequential image acquisition was performed. The absolute number of labeled cells was quantified in the entire cerebellar neurogenic niche (according to Grandel et al., 2006). To account for inter-

individual difference in brain size, the data were adjusted for brain volume, which was estimated based on images of individual brain sections (ImageJ) and using the Cavalieri principle (Prakash et al., 1994). Statistical significance for mean values was assessed with an unpaired Student's t test, with significance level of $p < 0.05$.

Mathematical modeling

The system of linear differential equations describes the time-dependent kinetics of neural progenitor cells (NPCs) and their progeny (NB/GB) in the Cerebellum, based on the experimental data obtained:

$$\frac{dN_p(t)}{dt} = -\alpha(t)(p_3 - p_1)N_p(t) - \frac{1}{\tau_p} N_p(t) + Q_o(t), \quad (Eq. 1)$$

$$\frac{dN_d(t)}{dt} = \alpha(t)(2p_3 + p_2)N_p(t) - \frac{1}{\tau_d} N_d(t), \quad (Eq. 2)$$

where $N_p(t)$ and $N_d(t)$ are time dependent volume density of NPCs and NB/GB cells, respectively; $\alpha(t)$ is the rate of NPC division; p_1 , p_2 and p_3 are probability of symmetric and asymmetric channels of NPC divisions. The parameters τ_p and τ_d describe the lifetimes of NPCs and NB/GB cells respectively. The life-times τ_p and τ_d describe the cell removal processes due to other factors, for example, a sporadic “sudden death” or conversion. $Q_o(t)$ is the rate of division of NSCs, which are assumed to be the only source of NPCs. The total balance of all the division and removal

processes is included in Eq. 1 and 2. Significant simplification of equations occurs in the steady state mode, when daily numbers of NPCs and NB/GB cells are not changing with time and NSCs produce a constant amount of NPCs.

In the steady state mode:

$$\frac{dN_p(t)}{dt} = 0 ; \frac{dN_d(t)}{dt} = 0 \text{ and } Q_o(t) = Q_o = \text{const.}$$

Chapter 3.4: Results

Normal cell kinetics and migration in a neurogenic niche of a young mature zebrafish.

Investigation into cell replication kinetics and migration was accomplished using two S-phase markers and a 3-day pulse-chase paradigm. Based on our earlier findings (Chapter 2), the first marker, BrdU, was administered to three groups of fish at the time of the documented daily peak in S-phase in adult zebrafish brain, from ZT9 to ZT11 (immersion). Following this Day 0 treatment, groups of animals received the second marker, EdU, on each of three consecutive days. EdU was injected at ZT9 and fish were collected at ZT11 (Fig. 13).

The pulse-chase approach revealed cells labeled with BrdU only, EdU only, or those with colocalized presence of BrdU and EdU. The latter two types of cells were present almost exclusively in the niche, while the BrdU-only cells were found in both the niche and outside parenchyma. According to the design, the BrdU-only cells could represent NSCs

that went through S-phase on Day 0, but not on consecutive days, and the post-mitotic progeny of NSCs and, more likely, NPCs. EdU-only cells represented those undergoing S-phase on the day of sample collection (NSCs and NPCs). The co-labeled cells were those that have undergone S-phase on Day 0 and were repeating it on the day the brains were collected, and were thus considered to represent NPCs.

The overview of the raw data (Fig. 14) revealed that there is a steady decline of BrdU-only cells in the niche over a 3-day period. This is due to both the migration of the post-mitotic progeny into parenchyma and reduction in the number of the original NPCs that were in S-phase on Day0 and thus were labeled by BrdU. This is reflected in the steep decline of co-labeled BrdU/EdU cells, representing NPCs. In contrast, a steep increase in EdU-only cells reflects that those expiring NPCs are replaced by new NPCs.

Accordingly, on Day 1 (24h), these EdU-only cells are likely to be represented by a new set of NSCs that are activated to produce new NPCs by Day 2 (48h). On days 2 and 3, the EdU-only cells already represent both the NSCs of that day and NPCs originating from a previous day (since BrdU label was not available beyond day 0 and thus absent in these new NPCs). The perfect balance between these two populations of cells and consistent day-to-day cell proliferation in a healthy young neurogenic niche is highlighted by the total number of EdU-labeled cells remaining stable (i.e., EdU-only plus co-labeled BrdU/EdU cells), with a mean of 1304 ± 72 cells over the three days studied (see also Fig. 22A, in Chapter 3).

The majority of NPCs progeny, NB/GBs, are leaving the niche between the 24 and 48h after the original Day 0 when labeling with BrdU occurred (Fig. 14C). The remaining BrdU-only cells (~400 cells daily) represent the NSCs of Day0 that remain quiescent, thus retaining BrdU and not acquiring EdU. Other BrdU-only cells are the remaining NB/GB, gradually leaving the niche.

Quantitative evaluation of the cerebellar neurogenic niche in young healthy adult zebrafish using mathematical modeling.

Understanding complex dynamic biological phenomena requires mathematical modeling. These models provide quantitative description of individual components of the evolving biological system and their interactions. Accurate modeling, if based on general principles of the specific system, allows for creation of not only a real-time picture of the system's behavior but predicts its evolution and response to changes in internal or external conditions. Based on the principles of cell behavior in neurogenic niche, as described above, and balance of its critical components, the experimental data collected as part of this dissertation was subjected to mathematical model procedures (see Methods). Those were based on the parameters fitted to only the data points for colocalized cells (Fig. 14). The other two curves were generated automatically, and then compared to the data for BrdU-only and EdU-only cell groups, resulting in a perfect fit (Fig. 15).

For the purpose of this investigation and based on the current understanding of the evolution of the neurogenic niche system composed of neural stem cells (NSCs) and transient amplifying neural progenitor cells (NPCs), it was accepted that NSCs divide infrequently and predominantly asymmetrically, producing one NSC and one transient amplifying cell, NPC. Accordingly, following a completed cell division cycle, most NSCs would remain in G0 for some time, before being activated. For the transient amplifying cells, NPCs, the daily division and its three potential modes were taken into account, i.e., the two symmetric ones resulting in two NPCs or two progeny (NB/GB), or asymmetric, producing one NPC and one progeny. The progeny would then be expected to migrate out of the niche and to be found in the outside brain parenchyma, with some being eliminated through apoptosis. The niche of interest described in this study is the cerebellar niche, chosen for its well delineated anatomy, robust daily proliferation in zebrafish (see Chapter 2), and since it has been well described by others (Kaslin et al., 2009; Kaslin et al., 2013).

The resulting curves of the mathematical model fit perfectly with the experimental data (Fig. 15), suggesting that the model described the kinetics within the niche with high precision. It provided the following quantitative description of the system and its behavior (Table 1). The average number of NPCs present in the niche daily was twice higher than the number of dividing NSCs that define the rate of NPC production (851 vs. 400, respectively). This supported the notion that a large number of NPCs repeat the cycle the next day or for several more days. Indeed, the average lifetime of NPC in the

niche was found to be 2.1 day. This timing was largely defined by the NPC pool depleted through division (255 cells per day), with the rest of NPCs lost through conversion or sporadic and undefined “sudden death”. The contribution of the latter modes of NPC loss was substantially lower than that of the division mode. This is also reflected in the symmetric NPC division into two daughter cells being 30% more likely than the symmetric division into two NPCs. The progeny of NPCs, NB/GB, migrated quickly (0.6 days) out of the niche. Their overall lifetime, including outside the niche, on average, was 2 days. This is consistent with rapid loss of neuroblasts describe earlier, resulting in only a small portion of the newly acquired cells differentiating and incorporating into active neuronal circuits (Sierra et al., 2010).

Chapter 3.5: Discussion

The results of this investigation revealed that young healthy fish have stable neurogenic activity in the cerebellum that does not significantly vary from day to day. The data collected over 3 consecutive days of a pulse-chase experiment and based on it mathematical modeling provided a unique window into the process of the daily turnover of cells in the neurogenic niche of adult zebrafish, their lifetime, modes of division and their proportion, and the rate of cell loss and migration. Together, this documented a complex picture of balanced and precisely orchestrated cell renewal machinery of the neurogenic niche in a diurnal vertebrate.

The remarkably perfect fit of the mathematical model curves with the experimental data validates several of our original assumptions that were based on the earlier reports or our observations described in Chapter 2. For example, the model supports that NSCs are highly unlikely to be activated within several days of the previously completed cell cycle. It also agrees with NPCs replicating daily within their short lifetime of transient amplifier cells.

We find that NPCs' symmetric division into two daughter cells is 30% more likely than a symmetric division into two NPC, effectively depleting the NPC pool and generating a large number of postmitotic progeny. This defines a relatively low number of consecutive divisions by NPCs and their overall lifespan of 2.1 days, on average. A sporadic loss or conversion are much less likely processes and NPCs would live, on average, 3.7 days if not for the depletion through division. The fast turnover of cycling cells we find in zebrafish neurogenic niche is similar to that reported in mammals (Sierra et al, 2010).

Chapter 3.6: Tables and Figures

Figure 13. The schematic of pulse-chase experiment on the kinetics of proliferating cells in adult zebrafish cerebellum.

On Day 0, three groups of fish were exposed to BrdU in tank water over a 2-h period (ZT9-11), corresponding to peak in the number of cells in S-phase (Fig. 6). On each of 3 consecutive days, one group of fish was injected (i.p.) with EdU and sacrificed 2h later, at ZT11, i.e., 24-72h after BrdU exposure. Arrows: BrdU administration (red), BrdU washout (blue), EdU injection (green), sample collection (black). N=5-6 fish/time point, AB strain, 1 year old males. 14:10 light-dark cycle, grey area -dark. Zeitgeber time (ZT), ZT0 = lights-on time.

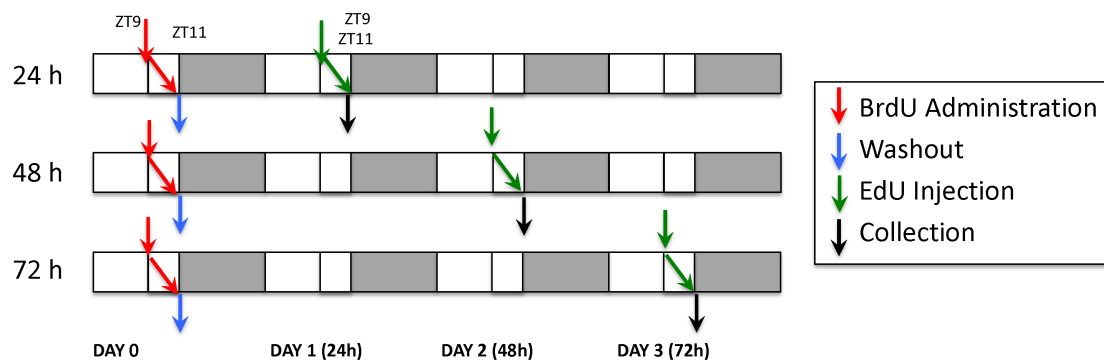


Figure 14. Change in the number of labeled cells following a 3-day BrdU/EdU pulse chase in the cerebellum of young healthy 1-year old fish.

A. Total number of cells in the neurogenic niche, *B.* Total number of cells in the whole cerebellum *C.* Percent of cells in the neurogenic niche vs. whole cerebellum. BrdU-only (red), EdU-only (green), BrdU/EdU colocalized (yellow). N=5-6 fish per time point, mean $\pm$ SEM.

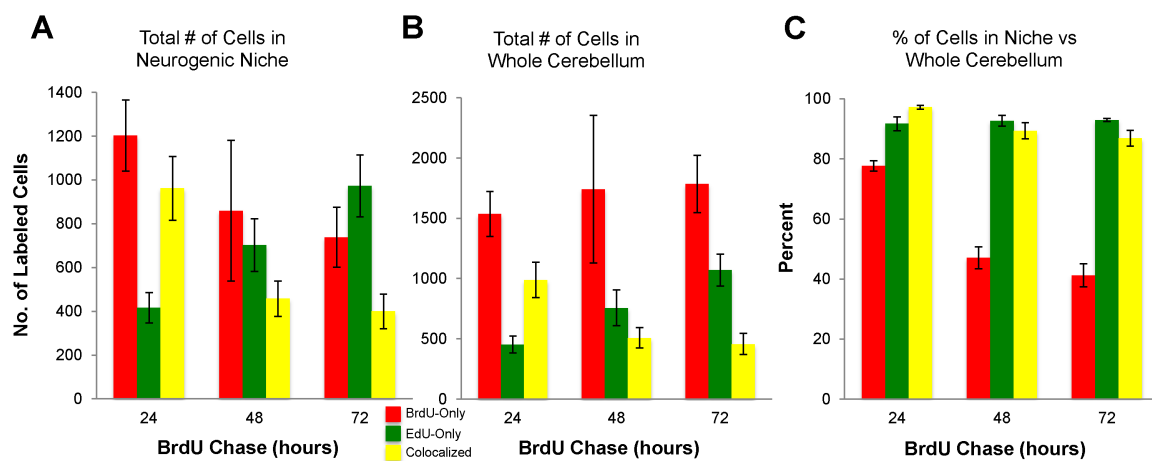
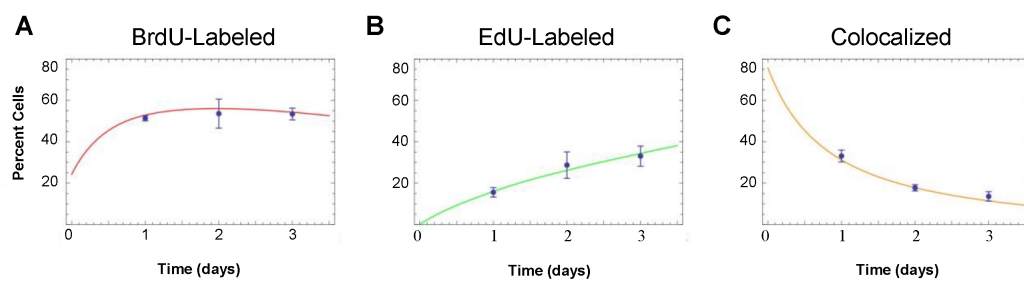


Figure 15. Result of mathematical modeling of the kinetics of three cell populations over time in young mature 1-year old fish.

A. BrdU-only cells *B.* EdU-only cells *C.* Colocalized BrdU/Edu cells.

Experimental results presented as mean % cells $\pm$ SEM of each label per day, based on the data in Fig. 14. Lines - mathematical model fit.



Estimated Parameters	1-year old Normal
No. of NSC in S, daily	400
No. of NPC in S, daily	851
NPC overall lifetime (days)	2.1
Rate of NPC production (#/day)	400
Rate of NPC loss/gain through NPC division (#/day)	(-)255
NPC "sudden death" (days)	3.7
NB/GB lifetime (days)	2
NB/Gb migration out of niche (days)	0.6
No. of NB/GB present in niche daily	612
No. of NB/GB leaving niche daily	1021
No. of NB/GB produced daily	1021
Mode of NPC division, p3 vs p1	p3 30%> p1

Table 1. Mathematical model-based parameters for the kinetics of NSCs, NPCs and their progeny in adult cerebellum, based on % cells of each label in the whole cerebellum over 3 days of pulse-chase experiment.

p1- symmetric mode of NPC division resulting in two NPC cells; p3-symmetric mode of NPC division resulting in two NB/GB cells.

CHAPTER FOUR: ALTERED ADULT NEUROGENESIS IN NORMAL AND PREMATURE AGING

Chapter 4.1: Abstract

This study documented the decline in adult neurogenesis in zebrafish during normal aging and revealed critical alterations in adult neurogenesis during pathologic premature aging. The model of premature aging was developed through excessive caloric load starting with early postnatal development and throughout maturation. This resulted in premature aging phenotype being reflected in altered body shape and behavioral patterns, as well as dramatic decline in the overall proliferation rate and cell kinetics within a neurogenic niche. Importantly, short-term changes in the quantity and quality of nutrition provided, and time of feeding, proved to have a profound effect on the magnitude of adult neurogenesis and its temporal pattern. Together, this opens new opportunities in using zebrafish to study the role of metabolic factors in accelerated aging processes, their impact on adult neurogenesis and interaction with the circadian regulation of neurogenic activity in adult diurnal vertebrate.

Chapter 4.2: Introduction

Aging is a normal but complex process influenced by multiple factors, including genetics, diet or stress, to name a few, leading to a high degree of interindividual variability in the pace of aging and symptoms associated with it. Aging can be characterized as

“successful” in those who with chronological age maintain relatively high physical and cognitive performance, or “unsuccessful” in those who show marked decline in these functions, often associated with age-related diseases. A separate category can be characterized as “premature aging”, where chronological age would predict a lack of the majority of aging-related changes, yet they are present. Recent evidence has suggested that the rate of aging is not fixed, but is plastic and open to modifications (Jellinger and Attems, 2013).

Neurogenesis can be a major contributor to neural plasticity in adult brain, along with synaptic modifications, and thus its alteration could play a significant role in the aging of the brain. While generation of new neurons continues throughout lifetime, there is a consistent age-related decline in the rates of adult neurogenesis across vertebrate species (for review, Lee et al., 2012). There is active debate whether this is a result of a decreasing pool of NSCs or due to an increase in their quiescence (Artegiani and Calegari, 2012). Clearly, more research is needed to elucidate the mechanisms behind aging capacity in neurogenic activity, changes in the mode of division of NSCs and NPCs, or survival of their progeny. This requires detailed analysis of cell kinetics and dynamics within aged neurogenic niches.

Through numerous studies on the pace of aging and factors that affect it, a diet and nutritional load were repeatedly found to be a major risk factor for accelerated aging. An unbalanced diet or overeating can lead to obesity which has been shown to accelerate age and age-related pathologies in humans and other species (for review, Haslam and James,

2005). Overnutrition, along with chronological age, have been implicated as prominent risk factors for neurological diseases, such as Alzheimer's and Parkinson's (Ballard et al., 2011; Beydoun et al., 2008; Hamer and Chida, 2009; Lu et al., 2009). Studies, so far, have shown compelling evidence that a high intake of dietary fat has a negative influence on hippocampal neurogenesis even in the absence of increased body weight and tissue accumulation (Lindqvist et al., 2006). In contrast, caloric restriction (CR), with a 30-40% reduction in the overall caloric intake is the only non-genetic intervention found to delay age-related cognitive decline and other disease states in mammals (Roth et al., 2001; Lin et al., 2002). CR in rodents was reported to increase neurogenesis in adult hippocampus and enhanced spatial learning and cognitive performance (Lee et al., 2000; Lee et al., 2002; Bondolfi et al., 2004). Furthermore, extending the time between meals, known as intermittent fasting, can increase neurogenesis (Stangl and Thuret, 2009). In zebrafish, so far, only one study addressed this issue, and reported that severe fasting for at least two weeks is detrimental to neurogenesis (Benitez-Santana et al., 2016). Together, these data suggest that the amount of food consumed and its quality are significant factors affecting adult neurogenesis and thus require in-depth investigation of the mechanisms involved.

Zebrafish provides an excellent model organism for studying the role of diverse intrinsic and extrinsic factors in the aging of neurogenic capacity. This is because they have very active neurogenesis at young age and, similar to humans, age gradually. Indeed, zebrafish can live up to 6-7 years under favorable conditions and exhibit clear morphological, anatomical and behavioral signs of successful or unsuccessful aging (Kishi et al, 2009).

While earlier studies have documented age-related decreases in neurogenesis in zebrafish up to 2.5 years of age (Edelman et al., 2013), no detailed characterization of this process has yet been conducted. This study thus provides the first in-depth investigation of the age-related decline in adult neurogenesis using two S-phase markers, allowing for pulse-chase approach, and complemented by mathematical modeling. Furthermore, we developed and validated a model of premature aging, based on overfeeding and increased caloric load throughout development and adulthood.

Chapter 4.3: Materials and Methods

Animals

Adult male zebrafish (*Danio rerio*, wild-type AB strain), 0.5-3 years-old were maintained on a 14 h light:10 h dark (14:10 LD) cycle, at 28°C, in 3L tanks of a multi-tank system (Aquaneering), as per standard practices (Whitmore et al., 2000). However, the normal and premature aging conditions were raised on different diets. Larval diet consisted of live food of *paramecium* and Type L saltwater rotifers (*Brachionus plicatilis*). Regular feeding schedule for mature fish consisted of twice a day administration at zeitgeber times (ZT) 1 and 8, (ZT0=lights on time) of pellets Gemma 300 (1.7% of body weight), with supplement live feed *Artemia salina* nauplii/metanauplii (brine shrimp). Premature aging fish were fed three times as much as healthy fish at ZT1 only, approximately 5% of their body weight, throughout larval and adult stages. Two months prior to experimental procedures, all fish were moved to regular feeding schedule of twice a day (ZT1 and 8).

All animal procedures were performed in accordance with Institutional Animal Care and Use Committee at Boston University School of Medicine.

Nutritional Value of Feed: Live Brine Shrimp and Gemma 300

Brine shrimp nauplii contain 37-71% protein, 12-30% lipid, 11-23% carbohydrate and 4-21% ash. The length of an average nauplius is 450 microns. Gemma (Skretting) feed is between 200-500 microns in size and contains 59% protein, 14% lipid, 0.2% fiber, 1.3% phosphates, and 14% ash.

Immunohistochemistry

Fish were euthanized with an overdose of MS222 (200 mg/l) and heads were fixed overnight in 4% paraformaldehyde in phosphate buffered saline (PBS) at 4°C. Brains were then dissected out, cryoprotected in 30% sucrose/PBS, placed in embedding solution (O.C.T) and stored at -80°C, until cut. Coronal 20 µm sections were prepared using a cryostat (Microm HM505E) and placed onto Fisherbrand Superfrost Plus slides (Fisher), then stored at -80°C, until processed. The sections were washed in 0.1 M PBS and antigen retrieval was performed using both heat, incubated in 50% formamide / 50% 2X SSC at 65°C for 2 h, and acid, 2 M HCl at 37°C for 30 min and 0.1 M boric acid (pH 8.5) for 10 min at room temperature. Following a PBS wash, the slides were incubated overnight in mouse anti-BrdU antibody directly conjugated to AlexaFluor 555 (Life Technologies, 1:20; in 0.1 M KPBS + 0.4% Triton-X100) at 4°C. After washing in PBS, sections were incubated in Click-iT® reaction cocktail for 30 min from the Click-iT®

EdU Alexa Fluor® 488 Flow Cytometry Assay (Invitrogen). After rinsing in PBS, slides were mounted using Vectashield mounting medium (Vector).

Microscopy and Analysis

Confocal microscopy was performed with a Zeiss LSM 710 using the Observer Z1 inverted microscope. Using Zen software, images were taken with a 20X objective. To minimize crosstalk between channels in multicolored tissue, sequential image acquisition was performed. The absolute number of labeled cells was quantified in the entire cerebellar neurogenic niche (according to Grandel et al., 2006). To account for inter-individual difference in brain size, the data were adjusted for brain volume, which was estimated based on images of individual brain sections (ImageJ) and using the Cavalieri principle (Prakash et al., 1994). Statistical significance for mean values was assessed with an unpaired Student's *t* test, with significance level of $p < 0.05$.

Locomotor activity recordings

To document locomotor activity patterns including the speed and depth at which fish were swimming, 1L tanks containing individual fish were placed on an experimental rack, with the lateral wall of each tank facing the camera. Walls of adjacent individual tanks were non-transparent white so fish were isolated and not influenced by their neighbor. The fish were habituated to tanks one hour prior to recordings and fluorescent lighting at 100 lx was provided equivalent that in their housing tanks. Activity in the entire tank (100 mm water column) and within 3 equally-spaced areas of the tank (the

top, middle and bottom) was documented for a 14-h period during the light cycle (normal day) using VideoTrack image analysis software (ViewPoint, Canada). The data acquisition speed was set at 30 frames/s. The speed (cm/s) and bottom dwelling (percent of time at bottom third) behaviors were documented for each condition. Behavioral studies involved 6-8 fish per group and all six aging conditions were documented in parallel. Consistent environmental conditions and thorough pre-recording calibration assured lack of recording artifact.

5-bromo-2'-deoxyuridine (BrdU) and 5-ethynyl-2'-deoxyuridine (EdU) Pulse-Chase Treatment

On Day 0, five groups of fish (1-year old premature aging) were exposed to BrdU in tank water for a 2-h period (ZT9-11), corresponding to peak in the number of cells in S-phase (see Chapter 2; Fig. 6). Following the 2-h period, the tanks were then washed out. On each of 5 consecutive days, one group of fish was injected (i.p.) with EdU (50 µg/g) and sacrificed 2h later, at ZT11, i.e., 24-120h after BrdU exposure (Fig. 16).

Diet, Time of Feeding, Fasting/Refeeding Conditions

There were 10 separate feeding regimens in this experiment (Fig. 17,18). If food was administered to the condition, it was given at ZT2 and ZT8 (ZT0= lights on time). Similarly, all fish were administered BrdU for a 24-h period at ZT3-3' corresponding to the trough in the number of cells in S-phase and the EdU injection (i.p.) at ZT9 corresponding to the peak in the number of cells in S-phase (see Chapter 2; Fig. 6). All fasting/refeeding conditions were fed live brine shrimp (Fig. 17).

The diet and time of feeding conditions began 96-h prior to collection (Fig. 18). The morning feeding condition was on fed at ZT1, while the evening feeding condition was only fed at ZT8, both were fed with live brine shrimp. The Caloric Restriction condition was fed half portions of live brine shrimp, the High Protein condition was fed Gemma Micro 300 (Skretting) and the Overfeeding condition was fed both Gemma Micro 300 and live brine shrimp.

Mathematical modeling

The system of linear differential equations describes the time-dependent kinetics of neural progenitor cells (NPCs) and their progeny (NB/GB) in the Cerebellum:

$$\frac{dN_p(t)}{dt} = -\alpha(t)(p_3 - p_1)N_p(t) - \frac{1}{\tau_p} N_p(t) + Q_o(t), \quad (Eq. 1)$$

$$\frac{dN_d(t)}{dt} = \alpha(t)(2p_3 + p_2) N_p(t) - \frac{1}{\tau_d} N_d(t), \quad (Eq. 2)$$

where $N_p(t)$ and $N_d(t)$ are time dependent volume density of NPCs and NB/GB cells, respectively; $\alpha(t)$ is the rate of NPC division; p_1 , p_2 and p_3 are probability of symmetric and asymmetric channels of NPC divisions. The parameters τ_p and τ_d describe the lifetimes of NPCs and NB/GB cells respectively. The life-times τ_p

and τ_d describe the cell removal processes due to other factors, for example, a sporadic “sudden death” or conversion. $Q_o(t)$ is the rate of division of NSCs, which are assumed to be the only source of NPCs. The total balance of all the division and removal processes is included in Eq. 1 and 2. Significant simplification of equations occurs in the steady state mode, when daily numbers of NPCs and NB/GB cells are not changing with time and NSCs produce a constant amount of NPCs.

In the steady state mode:

$$\frac{dN_p(t)}{dt} = 0 ; \frac{dN_d(t)}{dt} = 0 \text{ and } Q_o(t) = Q_o = \text{const.}$$

Chapter 4.4: Results

Age-Related Decline in Zebrafish Neurogenesis

Reduced neurogenesis in zebrafish with aging has been reported earlier (Edelman et al., 2013) but no detailed analysis of this phenomenon has been conducted. To accurately determine the age-related progression of changes in adult neurogenesis in zebrafish, we have compared animals at two young adult stages of maturation, 6-month and 1-year old, and then the 1-year old and 3-year old fish. This was based on EdU-labeled cells following a 2h exposure to this thymidine analog (i.p. injection) at the time of the peak S-phase in adult zebrafish, ZT9-11, as per our earlier evaluation (Akle, Stankiewicz et al., 2017; Fig. 6). Relative to 1-year control, the 6-month old fish showed equivalent number

of replicating cells (Fig. 19), while aged 3-year old fish demonstrated significant decline in proliferation (Fig. 20).

Zebrafish model of premature aging demonstrates dramatic decline in adult neurogenesis.

We then turned our attention to premature aging (PA), considering translational value of addressing this problem. As described in the Introduction to this Chapter, excessive caloric load is one of the few identified factors of preventable acceleration of aging, and CR is so far the only universally accepted method to delay aging process. We thus developed a model of premature aging (PA) in zebrafish through excessive caloric exposure starting early postnatal development and into young adult stages (10 months of age), followed by normal fish maintenance for at least 2 months prior to evaluation.

The success of this approach was evident through multiple behavioral and physiological variables being altered in the PA fish. By 1-year of age, PA animals developed first anatomical signs of aging, such as scoliosis and muscle wasting, and these further progressed over the next year of life (Fig. 20A). Such changes are typical of 4-6 year old fish undergoing normal aging.

We then explored locomotor activity and behavioral signs of anxiety in PA animals by assessing swimming performance, measuring the speed at which animals swim during the active daytime phase, and the degree of bottom-dwelling. The latter, a percent time spent

at the bottom third of the tank, is known to reflect anxiety-like behavior and our laboratory has recently demonstrated a progressive increase in this behavior in zebrafish undergoing normal aging (Kaprzak et al., 2017). In 3-year old fish undergoing normal aging and 1- or 2-year old PA animals, there was a trend toward decreased speed of locomotor activity, when compared to 1-year old normal control (Fig. 20B). Moreover, the 2-year old unsuccessful premature aging fish displayed significantly lower speed of locomotor activity relative to both the normal 1-year old group and 1-year old PA animals. Compared to normal 1-year old control, the bottom-dwelling was increased in 1- and 2-year old PA groups, and in 2-year old animals undergoing normal aging (Fig. 20C).

Then we compared the rate of cell proliferation in the adult neurogenesis of 1- and 3-year old, with that in 1-year old PA fish. The results demonstrated a significant change in this physiological function over a course of normal aging, as per earlier studies, and a dramatically more pronounced effect during premature aging. Compared to 1-year old control, the number of cells in S-phase in 3-year old normal fish was at $43 \pm 7\%$ reduction and in 1-year old PA fish it was at $21 \pm 3\%$ (Fig. 21).

Quantitative evaluation of the cerebellar neurogenic niche in zebrafish with premature aging phenotype using mathematical modeling.

We then used experimental methods and mathematical modeling that allowed us to conduct detailed evaluation of cell kinetics in neurogenic niche of normal animals

(Chapter 3) to determine whether not only the absolute amount of neurogenesis is changed with premature aging but also its kinetics. Accordingly, the investigation into the kinetics of cell division and migration in a 1-year old PA animals was accomplished using pulse-chase approach with two S-phase markers administered over 5 consecutive days, as illustrated in Fig. 16, above. The results demonstrated that, unlike highly stable neurogenesis in young normal adults (Fig. 22A and Chapter 3), 1-year old PA fish have remarkably unstable neurogenesis (Fig. 22B). Moreover, robust circadian amplitude of the cyclin A mRNA pattern in control animals was in contrast to low amplitude and phase advance observed in PA fish (Fig. 22C). This was in striking contrast to the PA zebrafish displaying upregulation of p20 gene (Fig. 22D), an inhibitor of G1-S transition (see more on this in Chapter 2), and Per1, a core clock gene (Fig. 22E).

As Figure 23 illustrates, PA fish also had a strikingly visible increase in the number of new cells entering S-phase starting day 2 (EdU-only cells, green), relative to those that were repeating the cycle (co-labeled for EdU and BrdU, yellow). Together, these changes observed in PA animals suggested that they have a highly unbalanced and unstable neurogenic system, with some functions failing and others being upregulated, potentially as part of a compensatory response to a developing pathology of premature aging.

Such complex and unstable dynamic picture of a physiological process warranted a mathematical modeling approach, which was applied similar to that in normal animals, as described in Chapter 3. Indeed, it provided us with a detailed picture of a major distortion

in the kinetics of adult neurogenesis in animals undergoing premature aging. The results of the mathematical modeling further supported that PA fish have high day-to-day variability in neurogenic activity in the cerebellum, inevitably leading to a less than perfect fit of the model curves to experimental data (Fig. 24). Yet, the model was able to establish a number of kinetic parameters with a high degree of certainty (Table 2). This was in major contrast to the perfect model fit in normal animals of the same age (Fig. 15).

The results of mathematical modeling shown in Table 2 also revealed low number of cycling NSCs and NPCs per day, 73 and 90 cells, respectively. This is approximately 5.5 and 9.5 times lower than the number of NSCs and NPCs in 1-year old normal fish having 400 active NSCs and 851 NPCs present daily (Table 1). Importantly, PA and normal fish display contrasting modes of NPC division. The rate of NPC gain/loss throughout the day was positive (+36 cells) in PA. This was a result of a 40% increase in the symmetric division producing two NPCs over that of producing two NB/GB progeny, which normally depletes NPC pool. Note a (-255) NPC loss per day in normal fish and a 30% higher chance of a symmetric cell division generating two NB/GB progeny cells in normal animals (Table 1). In spite of this phenomenon of a relative overproduction of NPCs, the overall lifetime of these cells was substantially shorter in PA than control, 1.3 vs. 2.1 days. This was a result of increased “sudden death” of these cells, i.e., decline in the number of NPCs not associated with their division into neuro- or glioblasts. The model revealed that if NPC cells would not be depleted through division, they would live 0.8 days in PA animals, compared to 3.7 days in normal fish (Tables 1 and 2). Moreover,

compared to normal 1-year old animals, those with PA showed slower migration of NB/GB from the niche (2.2 vs. 0.6 days) and a longer NB/GB lifespan at the periphery (20 vs. 2 days).

Together, mathematical modeling based on the detailed experimental data collected in animals with premature aging suggested that a dramatic decline in adult neurogenesis in PA is associated with low activity of stem cells, followed by potentially compensatory increase in the generation of NPCs through symmetric amplification of these transient cells. However, other yet unknown factors characteristic of a neurogenic niche in PA animals or NPCs themselves significantly reduce the lifetime of NPCs produced, making this overproduction ineffective. Finally, the progeny of NPCs, NBs/GBs, also demonstrate altered behavior, migrating slower and surviving longer than in normal fish. It should be noted that although both the overall evaluation of the data collected in PA animals and mathematical modeling based on that data reveal major alterations in their adult neurogenesis, the precision of quantitative results was inevitably affected by inherent instability of adult neurogenesis in PA fish, reflected in a less than perfect fit of the model curves (Fig. 24).

Nutritional factors and time of food intake produce major modification of adult neurogenesis in zebrafish with premature aging phenotype.

To reveal an impact of premature aging on adult neurogenesis, animals raised on high nutrient intake were placed on normal diet at least 2 months before the experiments

described above were conducted, to avoid an acute effect of excessive caloric intake.

After major modification in the quantity and kinetic characteristics of adult neurogenesis were revealed, we decided to conduct in-depth evaluation of the effects of diet and time of food administration in these fish.

We thus investigated the effects of fasting, different diets and feeding schedules on fish with premature aging. Note that similar studies in normal fish of different age remain yet to be completed. To accomplish this, fish were swimming in a BrdU solution for 24 consecutive hours, starting ZT3 of day 1 and until ZT3 of day 2 (ZT0=lights on time). At ZT9, i.e., around the known time of peak in the number of cells undergoing S-phase in neurogenic niches of adult zebrafish (Akle, Stankiewicz et al., 2017), animals received EdU injection (i.p.) (Fig. 17, 18), and were returned to BrdU-containing fish tank until the end of the 24-period when brain samples were collected. This allowed us to evaluate the overall number of cells undergoing S-phase over a 24-h interval (BrdU-total labeling), the portion of these cells that have completed S-phase by ZT9 (BrdU-only cells), and the portion of cells that entered the S-phase later in the day, after ZT9 (co-labeled for BrdU and EdU).

The 1-year old PA control animals received regular diet of brine shrimp twice a day and demonstrated a typical low number of S-phase cells for these fish, as well as a similar number of cells completing S-phase before and after ZT9 (Fig. 24-27). Acute fasting, within the first 24h, significantly decreased the number of cells in S-phase (54% vs.

control; BrdU-total) (Fig. 25). This trend was maintained in the 48h and 72h fast conditions, though these results did not reach significance. When compared to 48-h fast, animals re-fed during the last 24 hours of this period (i.e., 24h fast, followed by 24h refeed, samples collected at 48h) demonstrated a dramatic rebound (154% vs. control) in the number of cells undergoing S-phase. In contrast, no such rebound occurred after fish were fasted for 48-h and re-fed over 48-72h period, with re-fed fish showing significant decline in neurogenesis under such conditions, 48% vs. control (Fig. 25).

Comparison of the number of cells that completed S-phase by ZT9 and those initiating S-phase thereafter (i.e., BrdU-only and co-labeled cells, respectively) revealed that in Control animals these two pools of cells were similar (Fig. 25). In contrast, other conditions tested tended to change this balance toward cells undergoing S-phase later in the day and thus labeled by EdU that was administered at ZT9. This effect reached significance for 48-h fast and 72-h fast conditions.

The timing of food intake, morning versus evening, can modify the pattern of adult neurogenesis.

We then addressed whether administering food once a day, either in the morning or evening, each time the whole daily portion of regular food (brine shrimp), could interfere with the neurogenic activity and its timing. The results presented in Fig. 26A show that morning feeding was not different from control, while evening feeding caused increase in cell proliferation in the neurogenic niche, though did not reach significance ($p=0.08$).

Similarly, the balance between cells undergoing S-phase before and after ZT9 remained

similar to control in the morning group but was significantly skewed towards late part of the day in fish receiving food only late in the afternoon (Fig. 26B).

The role of diet and excessive caloric load on adult neurogenesis.

The final set of experiments of this Chapter was devoted to the role the diet or amount of food might play in adult neurogenesis, following the same methodological approach described above and illustrated in Fig. 18. The results demonstrated that providing twice a day high caloric load (overfeeding) led to significant increase in the overall number of cells in S-phase over 24h, relative to control (Fig. 27A). The high protein diet showed tendency toward increasing proliferative activity in the neurogenic niche ($p=0.06$). In contrast, the caloric restriction group that received two half-portions provided at regular times remained similar to control. When the timing of cell proliferation was analyzed, it revealed that all three conditions significantly favored late-day S-phase initiation (Fig. 27B).

Chapter 4.5: Discussion

The data presented here supports earlier reports on the age-dependent decline in adult neurogenesis in zebrafish. Importantly, it demonstrates that excessive caloric load (overfeeding) from early postnatal stages of development to maturity leads to severe aging phenotype in chronologically young animals. This is reflected in body shape and behavioral changes being consistent with accelerated aging process. When animals with

premature aging due to excessive caloric load are maintained under normal conditions, they display dramatic reduction in adult neurogenesis. Apart from the over 4 times lower division rate of NSCs, they also demonstrate major modification of virtually every aspect of cell kinetics in the neurogenic niche. The low pool of transient amplifying cells, NPCs, have a short lifetime due to either unfavorable niche environment or intrinsic cellular factors. Potentially, to compensate this, NPCs are making an attempt to increase their pool through a self-propagating symmetric mode of division (Table 2). This occurs at the expense of their progeny, i.e., neuroblasts or glioblasts. In turn, this progeny is not only scarce but has a low rate of migration and, according to the model estimates, prolonged lifetime at the periphery, when compared to normal control.

Our attempt to investigate which factors affected adult neurogenesis in these animals experiencing premature aging suggested that the amount and type of nutrients animals receive can play a major role in the vitality of their adult neurogenesis. Fasting reduces the number of proliferating cells in the neurogenic niche within the first 24h.

Remarkably, no significant decline in the total number of S-phase cells could be documented after 48 and 72h of fasting.

Re-feeding following 24-h fast produced a disproportionate increase in S-phase cells in the neurogenic niche. This was in striking contrast to refeeding following 48-h fast which resulted in even lower cell division rates than under regular feeding conditions. The dynamic changes of the effects of fasting should be explored further, especially in view of the well-characterized effective modification of internal energy sources during fasting,

switching from carbohydrate to lipid metabolism. Interestingly, a recently published study in zebrafish did not see decreases in proliferation in their optic tectum until 2 weeks of fasting (Benitez-Santana et al. 2016). Moreover, the well-characterized ability of NSCs to enter quiescence under unfavorable conditions and remain in this state should be one of the primary targets of further investigations. Clearly, more work is needed to elucidate the entire picture of the way neurogenesis is affected by multiple metabolic factors and their dynamic interactions.

Chapter 4.6 Tables and Figures

Figure 16. BrdU pulse-chase with EdU over 24-120h period in premature aging (1-year old) fish.

On Day 0, five groups of fish were exposed to BrdU in tank water over a 2-h period (ZT9-11), corresponding to peak in the number of cells in S-phase (see Chapter 2; Fig. 6). On each of 5 consecutive days, one group of fish was injected (i.p.) with EdU and sacrificed 2h later, at ZT11, i.e., 24-120h after BrdU exposure. Arrows: BrdU administration (red), BrdU washout (blue), EdU injection (green), sample collection (black). N=5-6 fish/time point, AB strain, 1 year old males. 14:10 light-dark cycle, grey area -dark. Zeitgeber time (ZT), ZT0 = lights-on time.

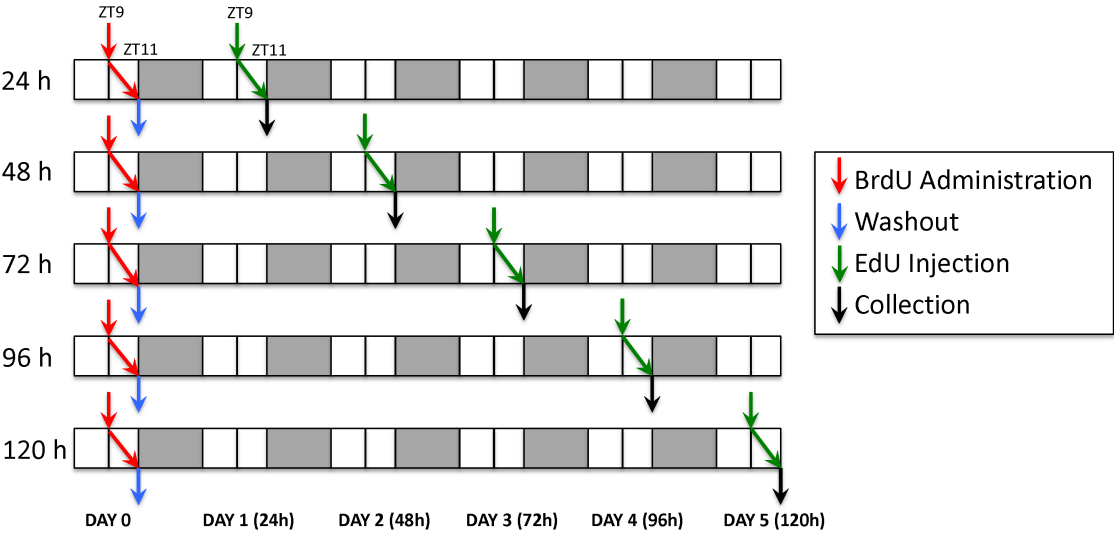


Figure 17. Fasting and refeeding conditions measured by 24h BrdU administration and EdU pulse in premature aging (1-year old) fish.

On Day 0, five experimental groups were exposed to different fasting and refeeding conditions maintained until collection. All fish were administered BrdU for a 24-h period at ZT3-3' corresponding to the trough in the number of cells in S-phase and the EdU injection (i.p.) at ZT9 corresponding to the peak in the number of cells in S-phase (see Chapter 2; Fig. 6) Arrows: Stop feeding (orange), refeeding (purple) BrdU administration (red), BrdU washout (blue), EdU injection (green), sample collection (black). N=5-6 fish/time point, AB strain, premature aging 1-year old males. 14:10 light-dark cycle, grey area -dark. Zeitgeber time (ZT), ZT0 = lights-on time.

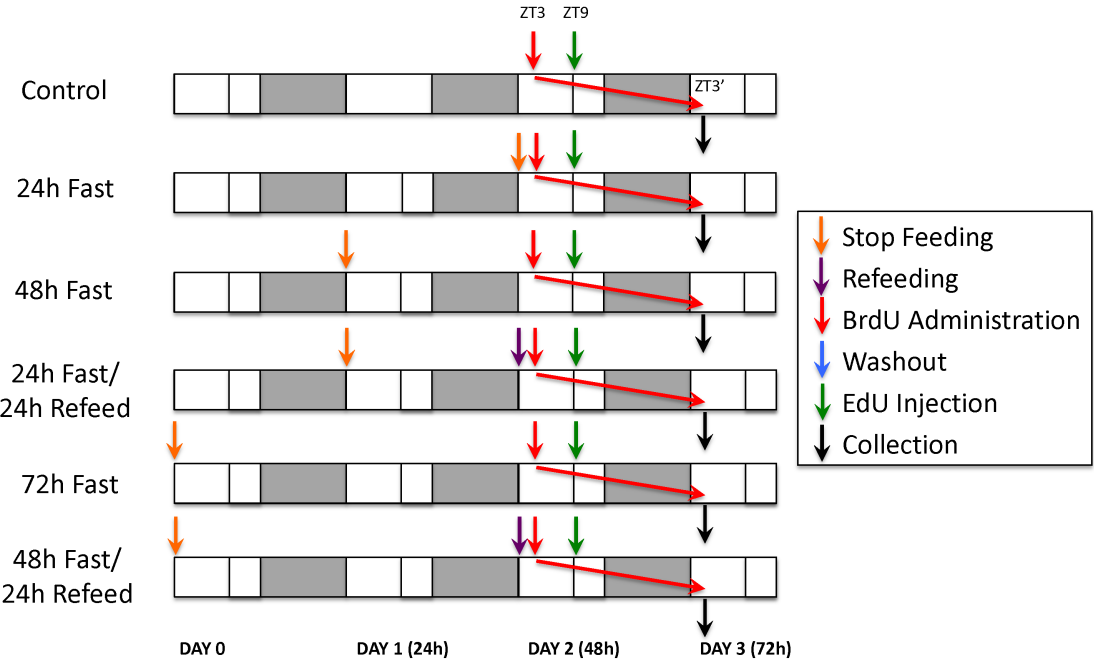


Figure 18. Time of feeding and diet measured by BrdU/EdU 24h pulse-chase in premature aging (1-year old) fish

On Day 0, five experimental groups were exposed to different feeding and diet conditions maintained until collection (96h). All fish were administered BrdU for a 24-h period at ZT3-3' corresponding to the trough in the number of cells in S-phase and the EdU injection (i.p.) at ZT9 corresponding to the peak in the number of cells in S-phase (see Chapter 2; Fig. 6). Control condition from Fig. 17. Arrows: Feeding protocol begins (teal), BrdU administration (red), BrdU washout (blue), EdU injection (green), sample collection (black). N=5-6 fish per condition, premature aging 1-year old males. 14:10 light-dark cycle, grey area -dark. Zeitgeber time (ZT), ZT0 = lights-on time.

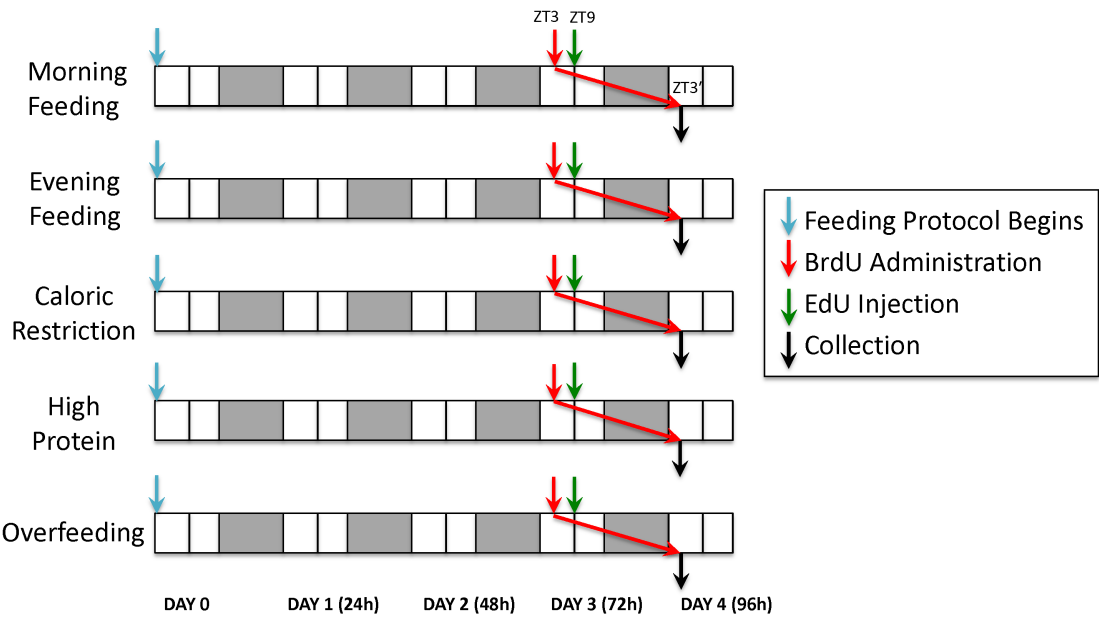


Figure 19. The absolute number of proliferating cells in the cerebellum of young adult 0.5-year old and 1-year old fish remains similar.

2-h EdU exposure via i.p. injection at ZT9. Number of cells in S-phase in the cerebellar niche at 0.5- (black) and 1-years of age (grey). N=5-6 fish per age, mean $\pm$ SEM.

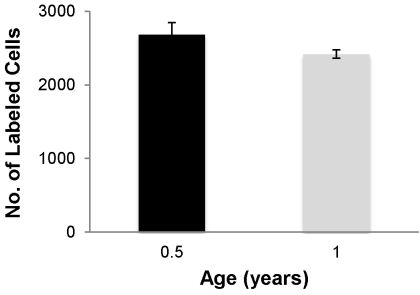


Figure 20. Changes in body shape and locomotor activity with chronological and premature aging.

A. a. 1-year old, normal; *b.* 1-year old, premature aging with scoliosis; *c.* 2-year old, normal; *d.* 2-year old, premature aging with scoliosis and muscle wasting. **B.** Age-related decline in speed of locomotor activity (cm/s) **C.** Bottom-dwelling, time spent swimming at the bottom third of the tank. Red bars - healthy fish (0.5 - 2-years of age). Blue bars: 1-year old with premature aging and 2-year old (“successful and unsuccessful” premature aging). N=6-8 fish per age, mean +/- SEM, *p<0.05 vs normal 1-year old.

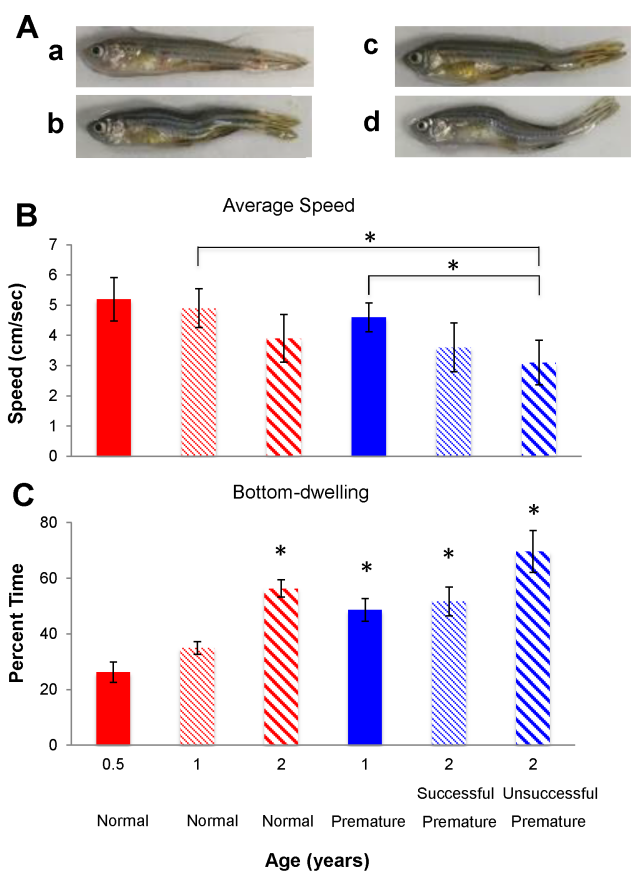


Figure 21. Reduction in adult neurogenesis with normal and premature aging.

Percent of cells in S-phase relative to healthy control 1-year old fish, expressed as 100%.

3-year old fish undergoing normal aging (red) and 1-year old fish with premature aging phenotype (blue). N=4-6/group mean $\pm$ SEM; * $p < 0.05$ vs 1-year old control.

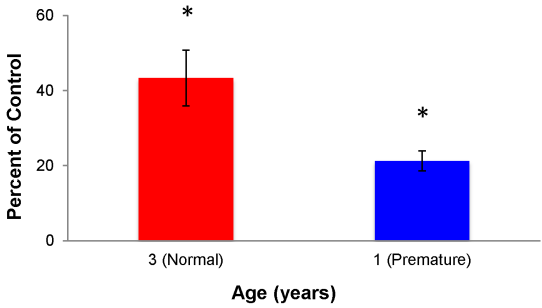


Figure 22. Modified process of adult neurogenesis in zebrafish with premature aging phenotype.

A. Consistent number of dividing cells in the cerebellum of normal 1-year old fish over 3 consecutive days; **B.** Day-to-day variability in the number of dividing cells in 1-year old fish with premature aging (PA). **C.** Dramatic decline in mRNA abundance and circadian amplitude of cyclin A in PA fish; **D.** Increase in mRNA abundance and circadian amplitude of p20 in PA fish; **E.** Increased amplitude in Per1 mRNA in PA fish. Red - Control, Blue- PA. Mean (SEM), N=5-6 fish/group (in A and B) or per time point in C-D. Grey background - night, 14:10 LD cycle. Zeitgeber time of sample collection, ZT0=Lights-on time.

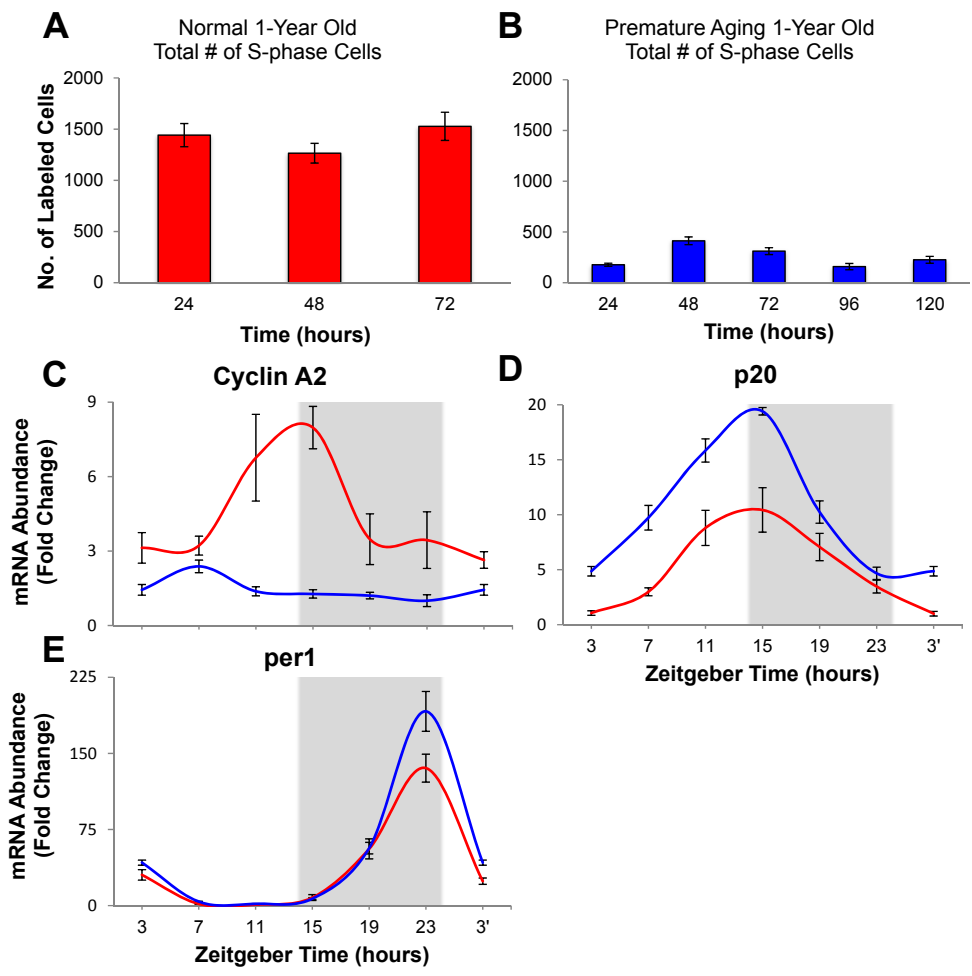


Figure 23. Change in the number of labeled cells following BrdU/EdU pulse chase in the cerebellum of 1-year old fish with premature aging.

A. Total number of cells in the neurogenic niche, *B.* Total number of cells in the whole cerebellum *C.* Percent of cells in the neurogenic niche vs. whole cerebellum. BrdU-only (red), EdU-only (green), BrdU/EdU colocalized (yellow). N=5-6 fish per time point, mean $\pm$ SEM.

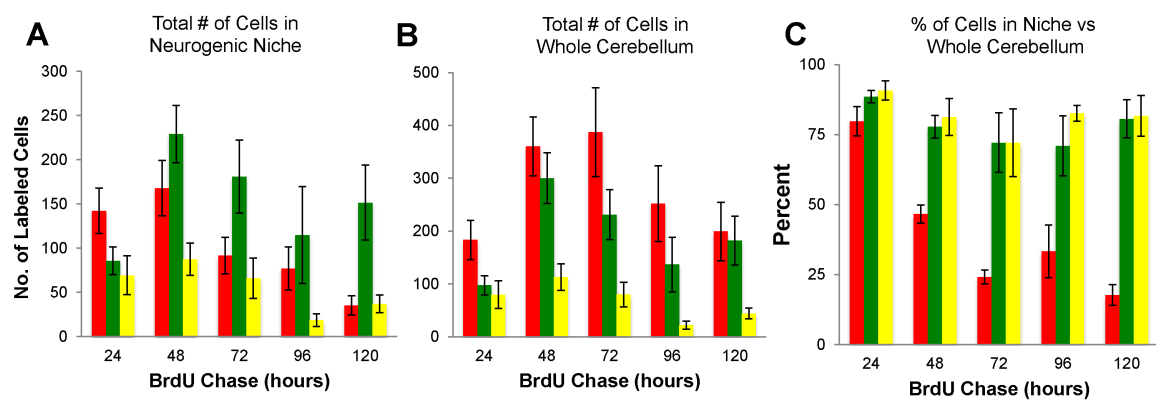
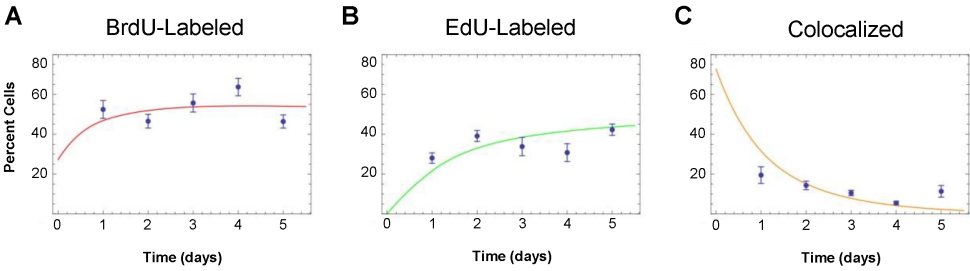


Figure 24. Result of mathematical modeling of the kinetics of three cell populations over time in 1-year old fish with premature aging.

A. BrdU-only cells *B.* EdU-only cells *C.* Colocalized BrdU/Edu cells.

Experimental results presented as mean % cells $\pm$ SEM of each label per day, based on daily data in Fig. 23.



Estimated parameters	1-year old Premature Aging
No. of NSC in S, daily	73
No. of NPC in S, daily	90
NPC overall lifetime (days)	1.3
Rate of NPC production (#/day)	73
Rate of NPC loss/gain through NPC division (#/day)	(+)36
NPC "sudden death" (days)	0.8
NB/GB lifetime (days)	20
NB/Gb migration out of niche (days)	2.2
No. of NB/GB present in niche daily	120
No. of NB/GB leaving niche daily	55
No. of NB/GB produced daily	55
Mode of NPC division, p3 vs p1	p3 <40% p1

Table 2. Mathematical model-based parameters for the kinetics of NSCs, NPCs and their progeny in adult cerebellum, based on % cells of each label in the whole cerebellum over 5 days of pulse-chase experiment.

p1- symmetric mode of NPC division resulting in two NPC cells; p3-symmetric mode of NPC division resulting in two NB/GB cells.

Figure 25. Effects of fasting and refeeding on neurogenesis in the cerebellum of 1-year old fish with premature aging.

A. Total number of BrdU-labeled cells in whole cerebellum in fish fasting for 24, 48 or 72 hours. Refeed: 24 hours of refeeding after 24h or 48h fast, samples collected at 48 and 72h, respectively. **B.** Percent of total number of cells completing S-phase before ZT9 (BrdU-only, red) vs. cells continuing S-phase beyond ZT9 (co-labeled for BrdU and EdU; yellow) within each treatment group. BrdU exposure over 24h (ZT3-ZT3'), followed by EdU injection at ZT9; N=4-5 fish per group, mean $\pm$ SEM, *P<0.05, relative to control.

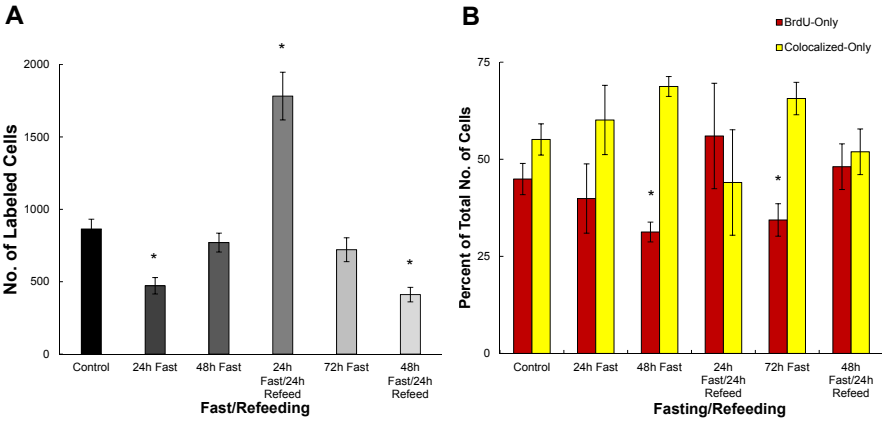


Figure 26. Effects of evening vs. morning feeding on neurogenesis in the cerebellum of 1-year old fish with premature aging.

A. Total number of BrdU-labeled cells in whole cerebellum in fish fed regular daily portion once a day, in the morning (ZT 2) or afternoon (ZT8). **B.** Percent of total number of cells completing S-phase before ZT9 (BrdU-only, red) vs. cells continuing S-phase beyond ZT9 (co-labeled for BrdU and EdU; yellow) within each group. BrdU exposure over 24h (ZT3-ZT3'), with EdU injection at ZT9; N=4-5 fish per group, mean $\pm$ SEM, *P<0.05, relative to control.

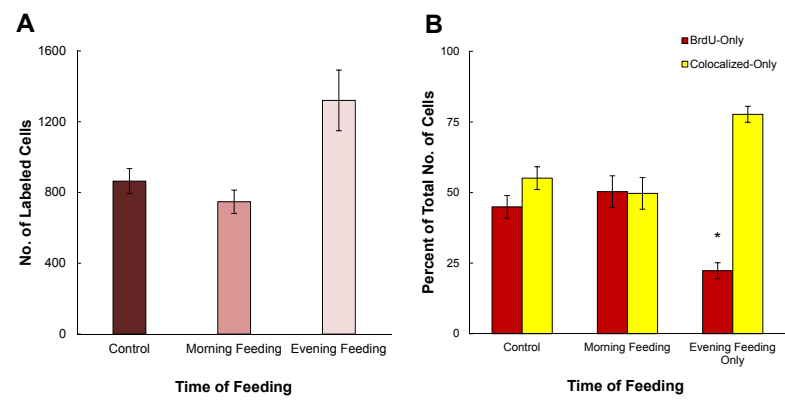
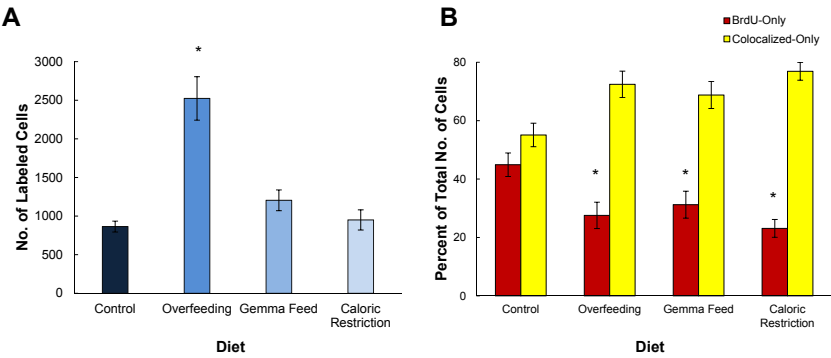


Figure 27. Dietary manipulations modulate neurogenesis in the cerebellum of 1-year old fish with premature aging.

A. Total number of BrdU-labeled cells in whole cerebellum in control fish (twice a day shrimp diet), overfeeding (twice a day double portion of shrimp and gemma), high protein (twice a day gemma), caloric restriction (twice a day half-a-portion of shrimp). **B.** Percent of total number of cells within each group completing S-phase before ZT9 (BrdU-only, red) vs. cells continuing S-phase beyond ZT9 (co-labeled for BrdU and EdU; yellow). BrdU exposure over 24h (ZT3-ZT3'), with EdU injection at ZT9; N=4-5 fish per group, mean $\pm$ SEM, *P<0.05, relative to control.



CONCLUSIONS AND FUTURE DIRECTIONS

While restricted to well-delineated neurogenic niches, adult neurogenesis is highly dynamic and modulated by numerous factors, intrinsic, pharmacological or environmental. Currently, there is lack of understanding of the exact temporal patterns of adult neurogenesis, kinetics of neural stem cells and transient progenitor cells, or neuroblast and glioblast migration. Animal models can provide unique insight into the time mechanisms of this phenomenon.

The research presented in this dissertation utilized major advantages of the zebrafish model to address some of these questions. Similar to humans, zebrafish is a diurnal vertebrate, and thus the two species share a similar temporal relationship between the circadian clock and the downstream clock-controlled processes. This is in contrast to nocturnal animals that have, relative to humans, an inverse relationship between these the circadian system and its targets. Zebrafish also have a robust circadian clock documented in all its tissues studied, including the brain. Finally, zebrafish have exceptionally active adult neurogenesis.

Using this model, the studies comprising this dissertation demonstrated for the first time that a diurnal vertebrate displays robust circadian pattern of adult neurogenesis. The orderly transition of cells from G1 to S phase of the cell cycle occurs throughout the day, followed by nighttime progression of G2 phase, and culminating with M phase in the early morning hours. Parallel analysis of neurochemical markers of adult neurogenesis,

based on incorporation of thymidine analogs BrdU and EdU, or G2/M marker pH3, was complemented by detailed evaluation of gene expression for critical intracellular regulators of the cell cycle, cyclins A, B and D, as well as p20, and inhibitor of G1-S transition. Together, the data revealed that the circadian pattern of adult neurogenesis is intrinsic, yet significantly augmented by periodic changes in environmental illumination.

This dissertation, through studying 5 neurogenic niches in parallel, has also established that these anatomically diverse niches of zebrafish brain are united by the common circadian pattern of cell proliferation, yet demonstrate distinct differences in the length of S-phase or the timing of surge in G1-S transition. This suggests that the unique microenvironment of each niche plays an important role in daily patterns of adult neurogenesis.

Further investigation into the kinetics of adult neurogenesis has focused on the events occurring in the neurogenic niches over days, revealing temporal patterns of maintenance and turnover of principal cells composing the niche. Studies described here, both experimental and involving mathematical modeling, determined that healthy young sexually-mature zebrafish have a consistent number of neural stem cells (NSCs) dividing daily, generating a consistent number of transient neural progenitor cells (NPCs). Moreover, it was found that in such normal adult fish the division modes for NPCs favor production of neuroblasts/glioblasts, relative to further propagation of NPCs. The NPCs make several rounds of division, 2 on average, while their progeny is rapidly leaving the

niche, translocating to the surrounding brain parenchyma, where the majority of them soon perish.

This dissertation also addressed age-related changes in adult neurogenesis in zebrafish, supporting its gradual decline in aged brains. To address pathological aging, a model of premature aging was developed based on excessive caloric load throughout development and maturation. Along with a premature aging phenotype being reflected in altered body shape and behavioral patterns, adult neurogenesis revealed dramatic changes in this model. Those involve a major decline in the number of dividing NSCs and extreme modification of the pattern of NPC division, producing only small number of progeny, while attempting to replicate themselves. Nevertheless, the lifetime of these relatively overproduced NPCs is so short for reasons unrelated to division, that the overall pool of their progeny remains extremely low. Remarkably, these progeny, neuroblasts/glioblasts are slow to leave the neurogenic niche and are predicted to survive in brain parenchyma much longer than those in normal fish.

Finally, the series of studies described in this dissertation addressed the extent to which feeding, including its nutritional value, content and timing of administration, can modify adult neurogenesis in animals undergoing premature aging. It was established that fasting over a 24-h period results in a major decline in adult neurogenesis, and is overcompensated upon refeeding. However, when fasting continues for up to 3 days, the process of neurogenesis appears to adapt to the lack of external energy sources. This

could be consistent with effective modification of internal energy sources known to occur during fasting, switching from carbohydrate to lipid metabolism. The data also suggest that the recovery from a more extended fasting period cannot be immediate, as we have observed a decline in neurogenic activity upon refeeding following a 48-h fasting.

Another interesting finding is that modifications of regular normal food-related parameters, including the amount of food, its content or timing of food availability, can modify a temporal pattern of cell division, favoring late-day S-phase transition. Clearly, zebrafish represents an outstanding model to explore the dynamic effects of metabolic modifications and diets on adult neurogenesis, and our future studies will address the broad range of applications that could stem from the current findings.

Together the results of the studies presented in this dissertation reveal that the process of adult neurogenesis undergoes predictable dynamic changes over hours, days and years, and that pathological conditions typical of humans, such as high caloric intake throughout development and maturation, can have a major impact on this process. Future studies using a high throughput zebrafish model should provide needed insights into the role of specific factors in adult neurogenesis and help in developing therapeutic strategies to benefit human patients.

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